CONTROL OF REGULATORY T CELL STABILITY, PLASTICITY AND FUNCTION IN HEALTH AND DISEASE

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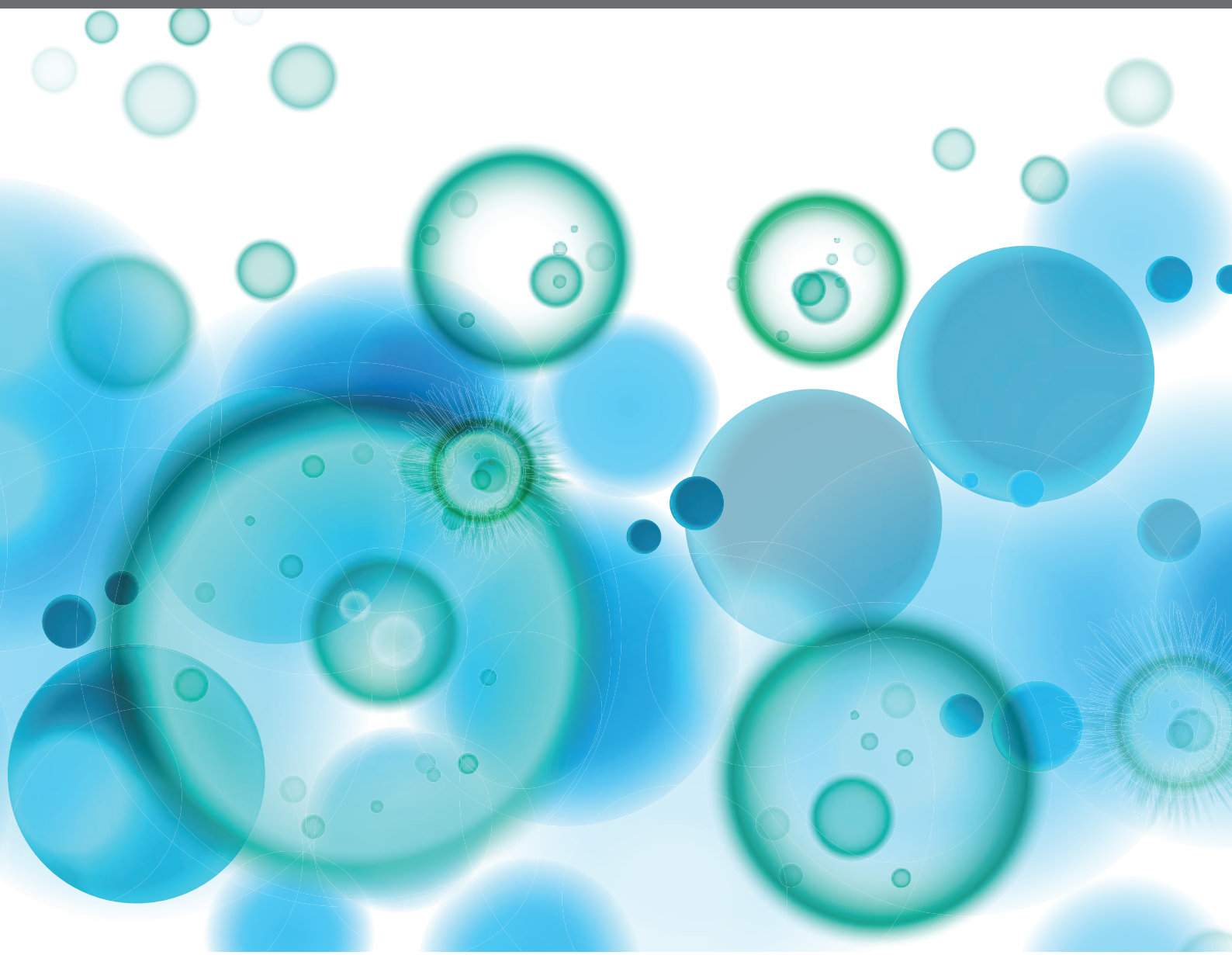

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CONTROL OF REGULATORY T CELL STABILITY, PLASTICITY AND FUNCTION IN HEALTH AND DISEASE

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frontiers in Immunology

EDITORIAL
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Editorial: Control of Regulatory T Cell Stability, Plasticity, and Function in Health and Disease

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Keywords: regulatory T cell (T reg), plasticity, autoimmunity, cancer, metabolism

Editorial on the Research Topic

Control of Regulatory T Cell Stability, Plasticity, and Function in Health and Disease

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Regulatory T cells ($CD4^+CD25^{high}CD127^-FOXP3^+$, Tregs) play a fundamental role in maintaining immune homeostasis by modulating the immune response against self-antigens, allergens, pathogens, and tumors. While Tregs were originally thought to be a terminally differentiated population of T cells whose only function was to inhibit the activation and/or proliferation of other immune cells, studies over the past decade have established that Tregs are a more plastic and dynamic population than previously thought and they have a far broader role mediated by their interaction with several immune and non-immune cells.

This Research Topic contains contributions that address the molecular mechanisms that regulate Foxp3 expression and Treg function, plasticity and instability, and the influence of environmental factors on these mechanisms in health and disease.

Tregs are a fairly stable population under homeostatic conditions, with tight regulation of the two major axes that establish the Treg program, i.e., Foxp3 expression and a Treg-specific epigenetic signature that is acquired during Treg development in the thymus (1). Stable and long-term expression of Foxp3 in Tregs is essential for Treg function and is partly controlled by the demethylation of Treg-specific epigenetic signature genes, including Foxp3 (2). Herppich et al. examine the dynamics of the imprinting of Treg-specific epigenetic signature genes in thymic-derived Tregs and demonstrate that the establishment of the Treg cell-specific hypomethylation patterns is a continuous process throughout thymic Treg development.

Regarding the regulation of Foxp3 expression and function, Zhang and Zhou review the evidence on Treg instability and the intrinsic and extrinsic mechanisms that control Foxp3 expression, proposing an interesting hypothesis that Foxp3 instability might help thymic derived Tregs distinguish between self and non-self antigens. In addition, Deng et al. review the post-translational modifications that control Foxp3 protein expression and therefore, Treg function,

and Colamatteo et al. review the mechanisms that control Foxp3 expression in healthy and autoimmune conditions.

Under inflammatory conditions, some Tregs can produce pro-inflammatory cytokines such as IFNγ and acquire an aberrant effector-like phenotype (plasticity) (3, 4) or even lose Foxp3 expression (instability) (5, 6). Such changes can be triggered in diverse settings including autoimmune, allergic, and infectious diseases (7). For example, patients with relapsing-remitting multiple sclerosis (MS), who display an increased frequency of IFNγ-producing Th1-like Tregs and a decrease in Treg suppressive function (3). Furthermore, a small percentage of Tregs in mouse models of MS have been shown to lose Foxp3 expression and become effector T cells, producing pro-inflammatory cytokines (IFNγ and IL-17) and contributing to disease severity (5, 6). In this regard, two reports in this book identify additional factors necessary to maintain Treg stability. Ronin et al. report that mice with a specific deletion of RelA in Tregs develop autoimmunity, which is attributable to the role of RelA in promoting Treg activation and stability, as RelA knock out Tregs lose Foxp3 expression and produce pro-inflammatory cytokines.

Di Giovangiulio et al. examine the involvement of the Tbet-IFNγ axis in colitis development. Tregs isolated from the lamina propria of active IBD patients display a Th1-like phenotype. Using a conditional Treg-specific Tbet KO, they observe that Tbet expression in Tregs is required for the development of colitis, and mice with Tbet KO Tregs develop milder colitis.

While these Treg plasticity and instability events are controlled by intrinsic molecular signaling pathways such as the PI3K/AKT pathway (8, 9), the activation of such pathways is modulated by the Treg environment, including cytokines, metabolic intermediates and dietary factors. As examples, Bin Dhuban et al. demonstrate that IL-27 and IL-6 signaling *via* gp130 impair the suppressive capacity of Tregs and render these Tregs unstable by downregulating Helios. Urbano et al. show that TNFα signaling through TNFR2 regulates the kinase architecture of activated Tregs and controls the expression of IL-17. Zhou et al. study the involvement of the HMGB1/PTEN/β-catenin pathway in myeloid cells in the development of Tregs during acute lung injury.

In regards to the influence of metabolism on Treg stability, Kempkes et al. review the metabolic profiles associated with the regulation of Treg functionality, and Shi and Chi provide a summary on the extrinsic and intrinsic metabolic factors and programs that regulate Treg lineage stability and plasticity, both in lymphoid and non-lymphoid tissues. Moreover, Arroyo Hornero et al. discuss the mechanisms underlying the effects of certain dietary components, including NaCl and fatty acids, on modulating Treg stability, plasticity, and function.

The tumor microenvironment is responsible for the specific phenotypes and functionality of infiltrating immune cells (10, 11). Toor et al. phenotypically characterize Tregs infiltrating tumor tissue in colorectal cancer patients and compare them to normal colon tissues and peripheral blood. They find an enrichment of highly suppressive Tregs ($Foxp3^+Helios^+$) in the tumor microenvironment, which upregulate a number of inhibitory receptors including CTLA-4, TIM-3, PD-1, and LAG-3. These receptors have been shown to modulate Treg function, migration, and proliferation (12).

Tregs can interact with non-immune cells and populate non-lymphoid tissues, where they perform non-immunological functions, mostly related to tissue repair and organ homeostasis (13). Korn and Muschaweckh and Sambucci et al. discuss the mechanisms that maintain Treg stability and function in non-lymphoid tissues, utilizing the central nervous system in the context of autoimmunity as an example. Albany et al. review the involvement of Tregs in cardiovascular disease and atherosclerosis and Brown et al. discuss the mechanisms that drive Treg adaptation to the environment and host tissues. In a perspective article, Martini et al. propose the intriguing hypothesis that Tregs have evolved under the pressure of mammalian pregnancy and lactation and tolerization to mammalian gut microflora. However, the Treg benefit turns into unwanted deleterious effects at advanced ages, when autoimmune and cardiovascular diseases and cancer can develop.

Tregs are a heterogeneous population and the biology underlying specific subpopulations is important to understand different roles of Tregs in various diseases. For example, Ding et al. give an update on follicular regulatory T cell biology with a particular focus on autoimmunity. Motwani et al. perform a deep characterization of cord blood *versus* adult blood Tregs and demonstrate that adult blood Tregs are a more heterogeneous population with less effector-like cells, and point to the use of expanded cord blood Tregs as a potential optimal adoptive cell therapy option for the treatment of autoimmune and inflammatory diseases.

Altogether, these works provide a comprehensive update on the immunological mechanisms underlying the control of Treg functionality, plasticity and instability, and the involvement of environmental factors in their modulation. This novel information on control of Treg stability, plasticity and function could help to guide the development of novel therapies to treat human disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial and direct intellectual contribution to the work, and all approved it for publication.

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Signaling Through gp130 Compromises Suppressive Function in Human FOXP3$^+$ Regulatory T Cells

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The CD4$^+$FOXP3$^+$ regulatory T cell (Treg) subset is an indispensable mediator of immune tolerance. While high and stable expression of the transcription factor FOXP3 is considered a hallmark feature of Treg cells, our previous studies have demonstrated that the human FOXP3$^+$ subset is functionally heterogeneous, whereby a sizeable proportion of FOXP3$^+$ cells in healthy individuals have a diminished capacity to suppress the proliferation and cytokine production of responder cells. Notably, these non-suppressive cells are indistinguishable from suppressive Treg cells using conventional markers of human Treg. Here we investigate potential factors that underlie loss of suppressive function in human Treg cells. We show that high expression of the IL-6 family cytokine receptor subunit gp130 identifies Treg cells with reduced suppressive capacity *ex vivo* and in primary FOXP3$^+$ clones. We further show that two gp130-signaling cytokines, IL-6 and IL-27, impair the suppressive capacity of human Treg cells. Finally, we show that gp130 signaling reduces the expression of the transcription factor Helios, whose expression is essential for stable Treg function. These results highlight the role of gp130 in regulating human Treg function, and suggest that modulation of gp130 signaling may serve as a potential avenue for the therapeutic manipulation of human Treg function.

Keywords: FOXP3, regulatory T cell, suppression, autoimmunity, immune regulation, gp130, cytokines

INTRODUCTION

CD4$^+$FOXP3$^+$ regulatory T cells (Treg) play an essential role in the maintenance of tolerance to self and harmless antigens. Congenital or acquired deficiencies in Treg cells result in severe autoimmunity in several animal models as well as in humans, and adoptive transfer of Treg cells controls autoimmunity in several animal models (1, 2). Numerous studies have examined potential defects in the Treg population as underlying or contributory factors in human organ-specific autoimmunity [reviewed in (2)]. While several groups reported numerical and functional defects in the Treg compartment in a number of autoimmune diseases such as multiple sclerosis (MS), type 1 diabetes (T1D), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), others have observed normal Treg frequency and function in these diseases [reviewed in (2)]. In addition to potential disease heterogeneity and methodological variations that may have contributed to the variable findings in these studies, lack of reliable human Treg cell markers is a significant limitation (2). While a number of markers allow for the detection of highly-enriched

Treg cells in resting conditions, most of these markers are inducible on effector T cells (Teff) upon TCR-mediated activation, thus leading to the inclusion of activated Teff contaminants and increasing the functional heterogeneity of the population (2). This results in considerable phenotypic and functional variability in the global Treg cell pool examined from healthy individuals and autoimmune patients, thus confounding the interpretation of results. Furthermore, FOXP3 is transiently upregulated in Teff cells upon TCR-activation without endowing them with suppressive function (3, 4), thus blurring the distinction between human Treg and activated Teff cells.

Using a single-cell cloning strategy that allows the discrimination between activated Teff contaminants and *bona fide* FOXP3-expressing Treg clones, we have recently shown that the human FOXP3$^+$ Treg population is functionally heterogeneous, containing a sizeable proportion of clones with an impaired capacity to suppress the proliferation of Teff cells despite exhibiting the hallmark surface phenotype of functional Treg cells (5, 6). We have further demonstrated that this FOXP3-positive, suppression-negative (FPSN) subpopulation, resembles its FOXP3-positive, suppression-positive (FPSP) counterpart in the demethylation status of the Treg-specific demethylated region (TSDR) of the *FOXP3* locus, as well as in the global Treg gene expression signature (6). These findings indicated that these non-suppressive FOXP3$^+$ cells likely originate from previously functional Treg cells. There are currently no markers to delineate these dysfunctional FOXP3$^+$ cells, and their prevalence and potential role in autoimmunity remains unknown. This study aims to characterize the factors that drive loss of suppressive function in human Treg cells, and to identify surface markers of dysfunctional Treg cells.

Several inflammatory mediators have been shown to modulate the function of Treg cells, including inflammatory cytokines such IL-1β, TNF-α, and IL-6, as well as several TLR ligands and microbial metabolites [reviewed in (7)]. The effects of IL-6 on Treg function have been particularly well-studied. IL-6 plays a critical role in regulating the balance between T helper 17 (Th17) cells and Treg cells, by favoring the differentiation of Th17 cells over Treg cells in the presence of TGF-β (8, 9). IL-6 has also been shown to inhibit *in vitro* and *in vivo* Treg-mediated suppression in mice (10–12) and humans (13). Clinically, elevated circulating levels of IL-6 are detected in the sera and urine of SLE patients, and correlate with disease severity (14). IL-6 is also highly elevated in the synovia of RA patients (15), and in the intestinal mucosa of inflammatory bowel disease (IBD) patients (16). Blockade of IL-6 using tocilizumab, an approved treatment for RA and other autoimmune disorders, has been shown to correlate with increased frequency of Treg cells, although Treg function was not assessed in these settings (17–20).

IL-6 signals through a receptor complex comprised of IL-6R (CD126) and gp130 (CD130) (21). Gp130 is part of the receptor complex for several cytokines, including IL-6, IL-27, IL-11, Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF), Cardiotrophin 1 (CT-1), and Cardiotrophin-like Cytokine (CLC) (22). The gp130 receptor is ubiquitously expressed on hematopoietic and non-hematopoietic cells, and its deletion in mice is embryonically lethal due to defects in cardiac development (23). However, postnatal conditional abrogation of gp130 in hematopoietic cells results in impaired lymphocyte development (24).

IL-27 is a cytokine of the IL-12 family. It is a heterodimer composed of the IL-27p28 and the Epstein-Barr virus induced 3 (Ebi3) subunits, and is produced by activated antigen-presenting cells (APC) such as dendritic cells and macrophages (25). IL-27 signals through the IL-27 receptor complex comprised the IL-27RA (WSX-1) and gp130 (25). Both pro- and anti-inflammatory roles have been described for IL-27. As a pro-inflammatory cytokine, IL-27 has been shown to induce the production of IFN-γ and favor the differentiation of Th1 cells in a STAT1-dependent manner (26–28). Furthermore, IL-27 interferes with TGFβ-induced generation of Treg cells (29), and more recently, Zhu et al. reported that IL-27, delivered using an adeno-associated virus (AAV)-based system results in a rapid depletion of Treg cells and enhances anti-tumor responses in a mouse model of melanoma (30). On the other hand, IL-27 has been shown to increase the production of IL-10 by effector CD4$^+$ and Tr1 cells (31–33), and to attenuate Th17-mediated inflammation in the EAE model (33–35). Furthermore, some groups have reported a paradoxical role for IL-27 in potentiating the suppressive function of Treg cells (36).

In this study, we investigated factors that drive loss of suppressive function in human FOXP3$^+$ Treg cells. We found that expression of gp130 identifies Treg cells with reduced suppressive function directly *ex vivo*. Furthermore, we show that IL-6- and IL-27-mediated signaling through gp130 impairs the suppressive capacity of Treg cells. These results highlight the important role of gp130-signaling in modulating the suppressive function of human Treg cells and present a novel target for the therapeutic modulation of Treg function.

MATERIALS AND METHODS

Donors and Cell Isolation

Peripheral blood mononuclear cells (PBMC) were purified from buffy coats of healthy donors (Sanguine Biosciences) using Ficoll-Paque PLUS density gradient (GE Healthcare), and were cryopreserved.

Reagents

Cryopreserved PBMCs were thawed and stained with viability dye (eFluor 780; eBioscience). Antibodies against CD4 (FITC or V500), CD25 (APC), CD45RA (Alexa Fluor 700)(BD Biosciences), CD127 (PE-eFluor 610), FOXP3 (PE), TIGIT (PerCP-eFluor710) (eBioscience), Helios (Pacific Blue; Biolegend). Purified anti-FCRL3 antibody was provided by Nagata (37), and was detected with F(ab′)2 anti-mouse IgG (PE-Cy7; eBioscience). Flow cytometry analysis was performed on an LSR Fortessa analyzer (BD Biosciences), and sorting throughout this study was performed on a FACS Fusion cell sorter (BD Biosciences). Recombinant human IL-6, IL-27, CLC, IL-11 (R&D systems), and LIF (Peprotech) were added to suppression assays where indicated at the time of activation.

Generation of Primary CD4$^+$ T Clones From Human PBMC

Primary CD4$^+$ clones were generated from healthy donors by single-cell sorting of CD25^{High} and CD25^{Neg} cells as described previously (5, 6). The clones were stimulated with soluble anti-CD3 (30 ng/mL; eBioscience), recombinant human IL-2 (200 U/mL) and irradiated human PBMCs as feeders, and propagated in Xvivo-15 medium (Lonza) supplemented with 5% FBS (Sigma-Aldrich). Fresh medium and IL-2 was added on day 5 and every 2 days thereafter, and clones were passaged as required. Clones were re-stimulated on day 11–12 and further expanded until harvest on day 22–24. Phenotypic analysis and functional assessment of suppressive capacity were performed in parallel.

In vitro Suppression Assays

Suppression assays were performed as previously described (5). Briefly, responding allogeneic CD4$^+$CD25$^-$ cells (Teff) were FACS-sorted, stained with the CFSE proliferation dye (5 μM; Sigma-Aldrich) and plated at 8,000 cells/well in U-bottom 96-well plates (Sarstedt) with irradiated PBMCs as feeders (APCs) (30,000 cells/well). Treg cells were added to the culture at a ratio of 1:1 and the assays were stimulated with soluble anti-CD3 (30 ng/mL; eBioscience) for 4 days. In bead-stimulated suppression assays, cells were stimulated with anti-CD3/anti-CD28-coated beads at a 1:2 beads/cell ratio. APC-derived supernatants were generated from total healthy PBMCs stimulated separately with anti-CD3/anti-CD28-coated beads at a 1:2 beads/cell ratio. APC-derived supernatants were collected every 24 h for 3 days, and added immediately to the Treg-Teff co-culture.

Suppression values in all suppression assays were calculated based on the division index of Teff cells cultured in the absence of Treg cells using the following formula:

$$\left(1-\left(\frac{\textit{Division index of Teff cultured in the presnece of Treg cells}}{\textit{Division index of Teff cultured in the absence of Treg cells}}\right)\right)*100$$

Where indicated, recombinant human IL-6, IL-11, IL-27, CLC (all at 1-100 ng/mL), or LIF (100–1,000 ng/mL) were added to the suppression assay at the time of activation. When cytokines were added to a suppression assay, suppression at a specific cytokine dose was calculated based on the Teff cells culture in the absence of Treg cells in the presence of the corresponding cytokine at the corresponding dose.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 6.0 software. One-way ANOVA, followed by multiple-comparison testing was used to compare multiple (>2) groups, and the student *t*-test were used where indicated for two-group comparisons. A p-value of < 0.05 was considered significant.

RESULTS

APC-Derived Factors Drive the Loss of Suppressive Function in Primary Human Treg Clones

Although stable FOXP3 expression is considered a specific feature of Treg cells, it is now established that human CD4$^+$ Teff cells can express FOXP3 upon activation, making them indistinguishable from Treg cells in activation or inflammatory contexts (3, 4). This activation-induced FOXP3 expression is transient and subsides within a few days of activation. To circumvent this issue, we have developed and exploited a single cell cloning strategy to pinpoint the phenotypic and functional status of individual cells in the heterogeneous FOXP3 expressing population. In this approach, we expand clones generated from single CD4$^+$CD25^{High} and CD4$^+$CD25^{Neg} primary T cells from human PBMC, and analyze their phenotypic and functional profiles at the end of a short-term activation cycle, thus allowing activation-induced FOXP3 expression to subside resulting in a state of immune quiescence. At the time of harvest, only Treg cell-derived clones maintain high FOXP3 levels, thus reliably eliminating contaminating Teff cells. Using this approach, we have previously shown that Treg clones harbor a population that is functionally impaired despite exhibiting the canonical phenotype of Treg cells including high and stable expression of FOXP3 (**Figures 1A,B**) (5, 6).

All assessments of suppressive capacity of FOXP3$^+$ clones were thus far performed in the presence of irradiated PBMCs as APC to provide co-stimulation in the suppression assay (5, 6). APCs are a major source of several inflammatory cytokines, some of which have been shown to alter the function of mouse and human Treg cells (7). Therefore, we asked whether the loss of suppressive function observed in FPSN clones is driven by inflammatory factors provided by the APCs. To assess this hypothesis, we examined the suppressive capacity of FOXP3$^+$ Treg clones in the presence or absence of irradiated APCs. Interestingly, the lack of APCs in the co-culture almost completely rescued the suppressive function of FPSN clones (**Figure 1C**). The absence of APCs had no significant effect on the suppressive potency of the already suppressive FPSP clones, nor did it affect the lack of suppression in control FNSN clones (**Figure 1C**). To determine the nature of the APC-derived factors driving the observed Treg dysfunction, we examined the suppressive capacity of Treg clones in the presence of purified supernatant of activated APCs. APC-derived supernatant was sufficient to cause a significant reduction in the suppressive potency of FOXP3$^+$ clones (**Figure 1C**). These data demonstrate that soluble APC-derived factors contribute to the lack of suppressive function associated with FPSN clones.

Gp130, a Component of the IL-6R Complex, Is Preferentially Expressed in Non-suppressive FOXP3$^+$ Treg Cells

We have previously performed a whole-genome expression analysis on FPSP, FPSN, and FNSN clones in order to identify gene products that distinguish human Treg cells from

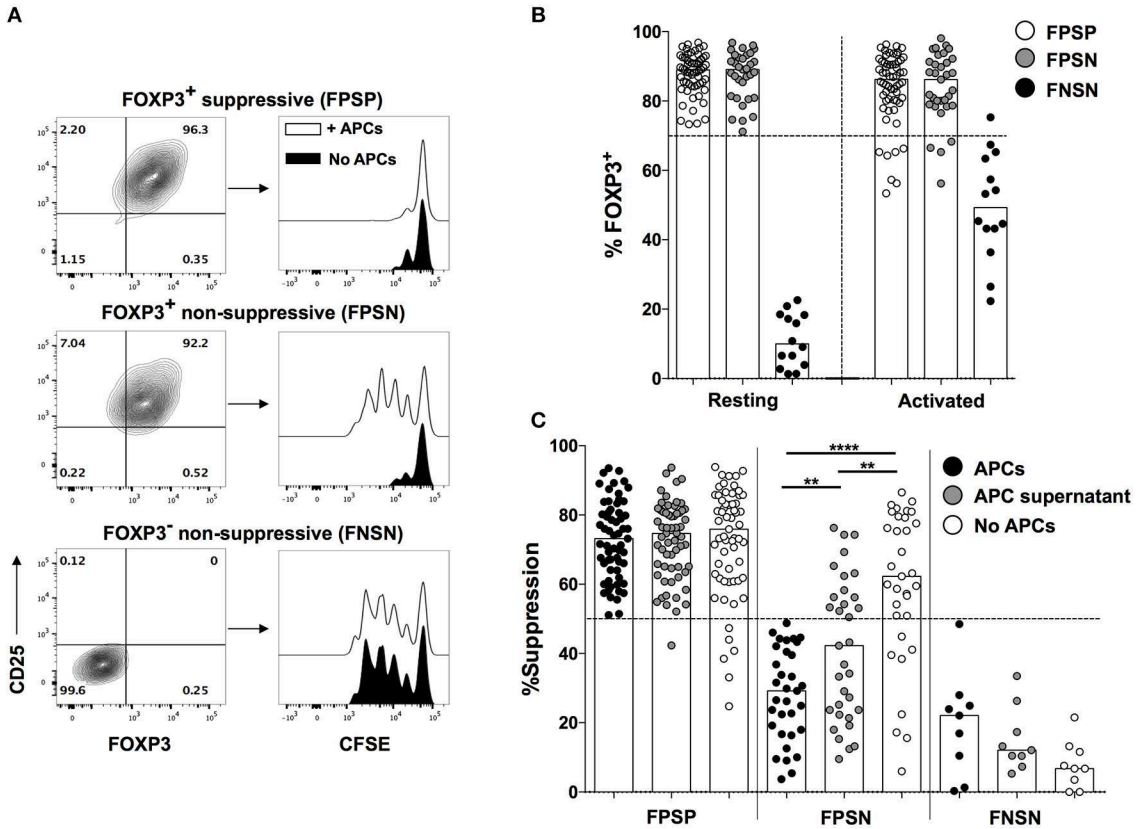

FIGURE 1 | Loss of suppressive function in FOXP3$^+$ Treg cells is driven by APC-derived factors. Primary FOXP3$^+$ clones were generated from healthy donors as described above, and were analyzed for their ability to suppress allogeneic CD4$^+$CD25$^-$ Teff cells in a 1:1 Treg:Teff suppression assay in the presence or absence of irradiated PBMCs (APCs). **(A)** Representative FACS plots showing CD25 and FOXP3 expression in representative FPSP, FPSN, and FNSN clones and their suppressive function in the presence or absence of APCs. **(B)** FOXP3 expression in FPSP, FPSN, and FNSN clones before, or 48 h after re-stimulation with anti-CD3 and irradiated APCs. **(C)** Suppressive potency of FOXP3$^+$ and FOXP3$^-$ clones in the presence or absence of APCs, or APC-derived supernatants. Shown are the suppression values for 61 FPSP, 31 FPSN, and 9 FNSN clones from one representative experiment of three different experiments where clones were generated from three different healthy individuals. Statistical analysis was done with the one-way ANOVA followed by the Tukey post-test. $^{**}p < 0.01$, $^{****}p < 0.0001$.

activated Teff cells and further identify the different functional subpopulations of Treg cells (6). Examining the expression levels of inflammatory cytokine receptors on the three populations, we observed an increased transcription level of the IL-6 receptor subunit (IL-6R) mRNA on both FOXP3$^+$ Treg subpopulations relative to FOXP3$^-$ controls in resting and activated conditions (**Figure 2A**). Although we did not observe a significant difference in IL-6R mRNA expression between FPSP and FPSN clones, we were prompted to further investigate the IL-6 pathway in the Treg clones due to the well-established role of IL-6 as a major antagonist of Treg function (10–13, 38). To that end, we generated FOXP3$^+$ and FOXP3$^-$ clones from CD4$^+$CD25^{High} and CD4$^+$CD25$^-$ cells, respectively, and identified FPSP, FPSN, and FNSN clones by examining their suppressive capacity in the presence of irradiated APCs. In parallel, we assessed the expression of IL-6R on the three subsets by flow cytometry prior to activation. Most examined clones expressed IL-6R, although no significant differences in IL-6R protein expression were observed among the three subsets (**Figure 2B**). Given the possibility of IL-6 trans-signaling in cells that do not express IL-6R (39, 40), we examined the expression levels of gp130 whose surface expression is required for IL-6 signaling. Interestingly, despite its mRNA being equally expressed by the three populations (**Figure 2C**), gp130 protein was significantly elevated on the surface of FPSN clones compared to FPSP and FNSN clones (**Figure 2D**), suggesting a potential role for this cytokine receptor in the functional impairment of the FPSN subset.

Gp130-Expressing Treg Cells Are Enriched Amongst the Naïve T Cell Compartment, and Display Features of Functional Instability

We next assessed the *ex vivo* expression of gp130 on total CD4$^+$ T cells and in Treg cells in healthy individuals. We found that gp130 is preferentially expressed on predominantly naïve (CD45RA$^+$) CD4$^+$ T cells, while the memory (CD45RA$^-$) compartment contains both gp130^{High} and gp130^{Low} cells (**Figure 3A**). A similar pattern of gp130 expression was observed in CD45RA$^+$ vs. CD45RA$^-$ Treg (CD4$^+$CD25$^+$CD127^{Low}) cells (**Figure 3B**). We have previously reported that Helios expression is associated

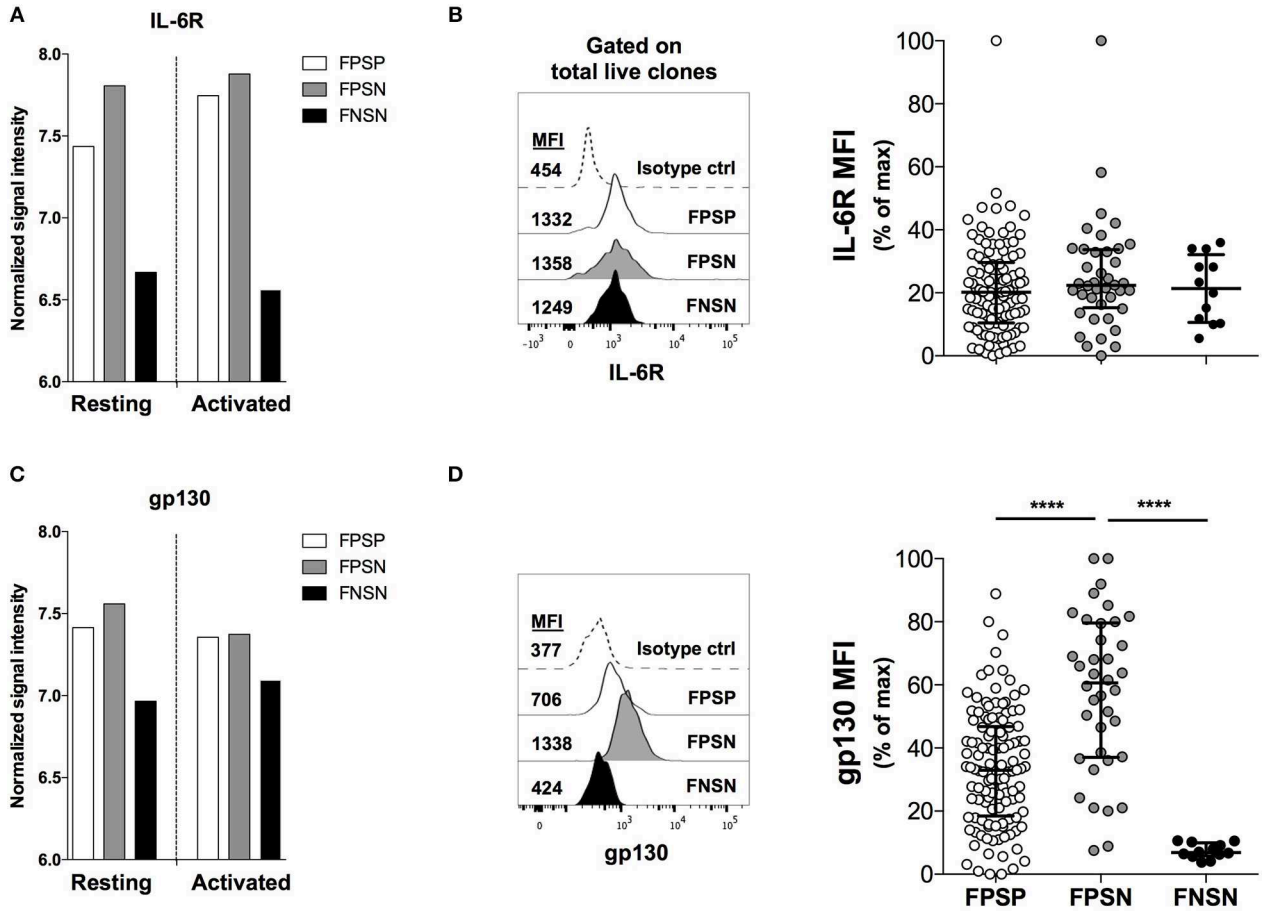

FIGURE 2 | Gp130, a component of the IL-6R complex, is preferentially expressed in non-suppressive FOXP3$^+$ clones. Primary FOXP3$^+$ clones were generated from healthy donors as described above. FPSP, FPSN, and FNSN clones were identified based on their ability to suppress allogeneic CD4$^+$CD25$^-$ Teff cells in a 1:1 Treg:Teff suppression assay in the presence of irradiated APCs. Shown are the relative mRNA expression levels of the IL-6 receptor chains IL-6R **(A)** and gp130 (IL-6ST) **(C)** in FPSP, FPSN, and FNSN clones as measured at the resting state at harvest or 24 h after activation (6). **(B,D)** show the protein expression levels of IL-6R **(B)** and gp130 **(D)** on FPSP, FPSN, and FNSN clones at the time of harvest. Data were normalized using the internal normalization feature in GraphPad Prism where the largest value in the data was set to 100 and the lowest value was set to 0. Shown are the results from one representative experiment of six different experiments where clones were generated from six different healthy individuals. Statistical analysis was done with the one-way ANOVA followed by the Tukey post-test. ****$p < 0.0001$.

with enhanced suppressive capacity and maximal repression of inflammatory cytokine production by human Treg cells (6). Moreover, we recently identified two surface proteins, TIGIT and FCRL3, as a reliable marker combination that distinguishes Helios$^+$ from Helios$^-$ Treg cells in human peripheral blood (6). We then investigated the expression of these novel surface markers in relation to gp130. Since both TIGIT and FCRL3 are preferentially expressed on memory Treg cells (6), while gp130 is highly expressed on CD45RA$^+$ Treg cells, we analyzed the expression of these markers on naïve and memory Treg subpopulations, identified based on CD45RA expression, and further distinguished based on gp130 expression (**Figure 3B**). As previously reported, FOXP3 expression is lower in naïve Treg cells relative to memory Treg cells (41). At the steady state, we did not observe a significant difference in FOXP3, Helios or TIGIT protein expression levels between gp130^{High} and gp130^{Low} within the memory Treg subset (**Figure 3B**). However, the frequency of FCRL3$^+$ cells is significantly reduced within the memory gp130^{High} Treg cells (**Figure 3B**). Thus, given the correlation between FCRL3 expression and stable Treg cell function (6), this reduced expression of FCRL3 could be indicative of reduced stability of gp130^{High} Treg cells.

Ex vivo gp130 Expression Identifies Treg Cells With Reduced Suppressive Capacity

We next sought to examine the correlation between gp130 expression and suppressive function of Treg cells directly *ex vivo*. To that end, we FACS-sorted the following 3 subpopulations of CD4$^+$CD25^{High}CD127^{Low} Treg cells: (1) CD45RA$^-$gp130^{Low}, (2) CD45RA$^-$gp130^{High}, and (3) CD45RA$^+$gp130^{High}. Given the high expression of gp130 on all naïve CD4$^+$ T cells, the fourth subpopulation CD45RA$^+$gp130^{Low} is almost non-existent and, therefore, we did not include it in the analysis (**Figure 4A**).

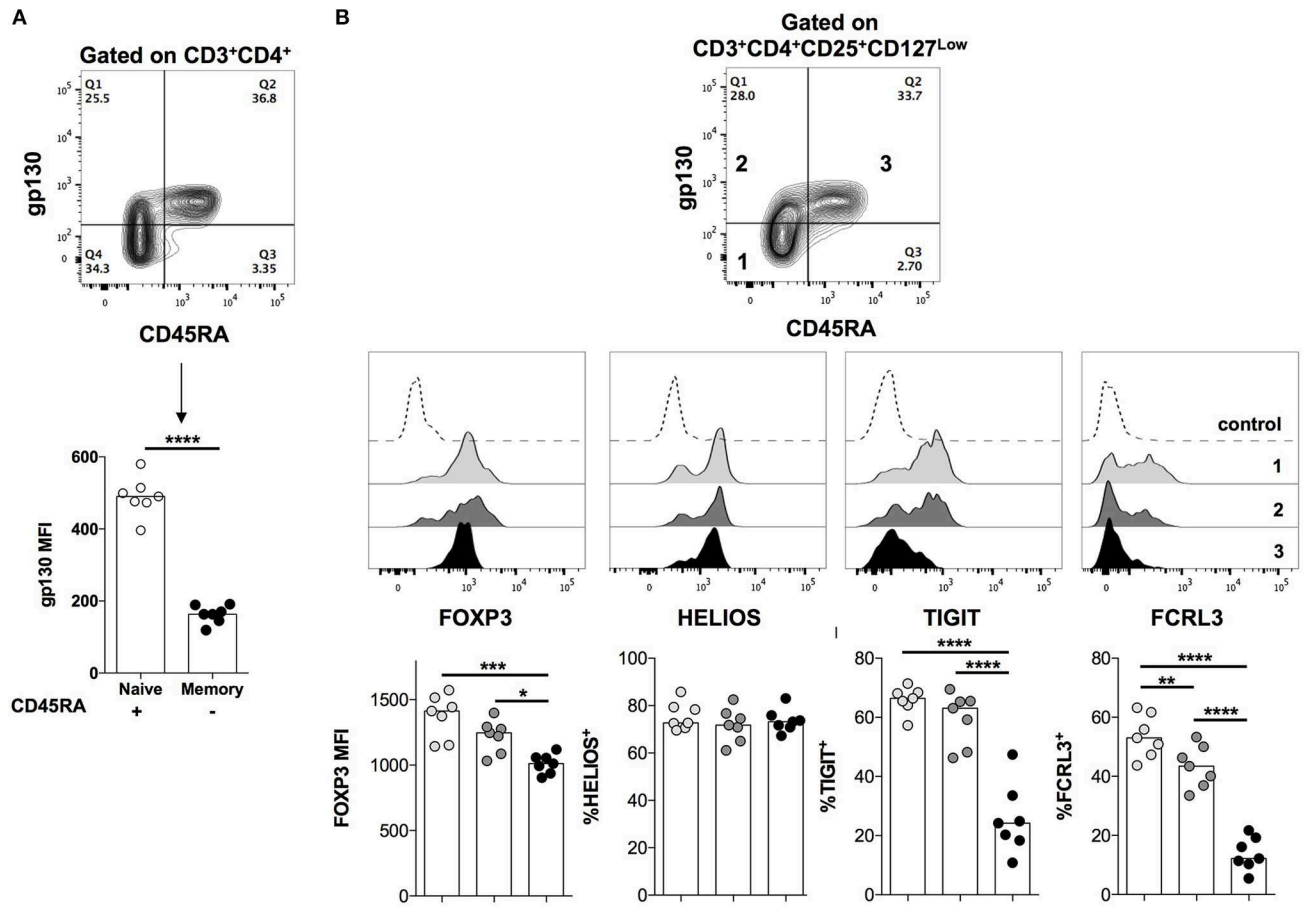

FIGURE 3 | Gp130 is preferentially expressed on naïve CD4$^+$ T cells. PBMCs were isolated from healthy individuals and analyzed *ex vivo* by flow cytometry for the expression of the indicated markers. **(A)** The expression of gp130 on naïve vs. memory CD4$^+$ T cells. Statistical analysis was performed using the student *t*-test. **(B)** The expression of gp130 on naïve vs. memory Treg cells and its correlation with the expression of FOXP3, Helios, TIGIT, and FCRL3. Shown are the combined results from seven different healthy individuals. Statistical analysis was done with the one-way ANOVA followed by the Tukey post-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Within the memory Treg population, we found that the suppressive potency of memory gp130^{High} cells was significantly lower than that of gp130^{Low} cells, and was comparable to that of naïve Treg cells, which have previously been reported to have a greatly reduced suppressive capacity in comparison with memory Treg cells (**Figure 4B**) (41). These results confirm our findings in Treg clones that gp130 identifies Treg cells with reduced suppressive capacity, and suggest that gp130 likely transmits inflammatory signals that dampen the suppressive capability of Treg cells.

IL-6 and IL-27 Inhibit the Suppressive Function of FOXP3$^+$ Treg Cells *ex vivo*

Several cytokines utilize gp130 as part of their receptor complexes, activating various downstream signaling pathways and driving different biological processes (42). Here we sought to identify gp130-signaling cytokines that alter the suppressive function of Treg cells. We generated primary Treg clones and assessed their capacity to suppress the proliferation of Teff cells in the presence of the gp130-signaling cytokines IL-11, LIF or CLC, IL-6, and IL-27. We first analyzed the effects of these cytokines on the proliferative capacity of responder Teff cells TCR-activated in the absence of Treg cells. We observed that only IL-6, but not the other tested cytokines, significantly increased the proliferation of Teff cells (**Figure 5A**). We next examined the suppressive potency of Treg clones in the absence or presence of exogenous cytokines. While the suppressive potency of Treg clones was not altered upon the addition of exogenous IL-11, LIF or CLC, both IL-6, and IL-27 markedly decreased Treg-mediated suppression of Teff cell proliferation (**Figure 5B**). However, while IL-27 exhibits a significantly higher modulatory effect on gp130^{High} relative to gp130^{Low} clones, IL-6 alters the suppressive function of gp130^{High} and gp130^{Low} clones to a similar extent, suggesting that IL-6 may additionally alter Treg-mediated suppression through the enhancement of Teff proliferation (**Figure 5A**).

We next assessed the impact of exogenous IL-6 and IL-27 on the suppressive function of Treg cells *ex vivo*. To that end, we sorted CD4$^+$CD25$^+$CD127^{low} Treg cells and

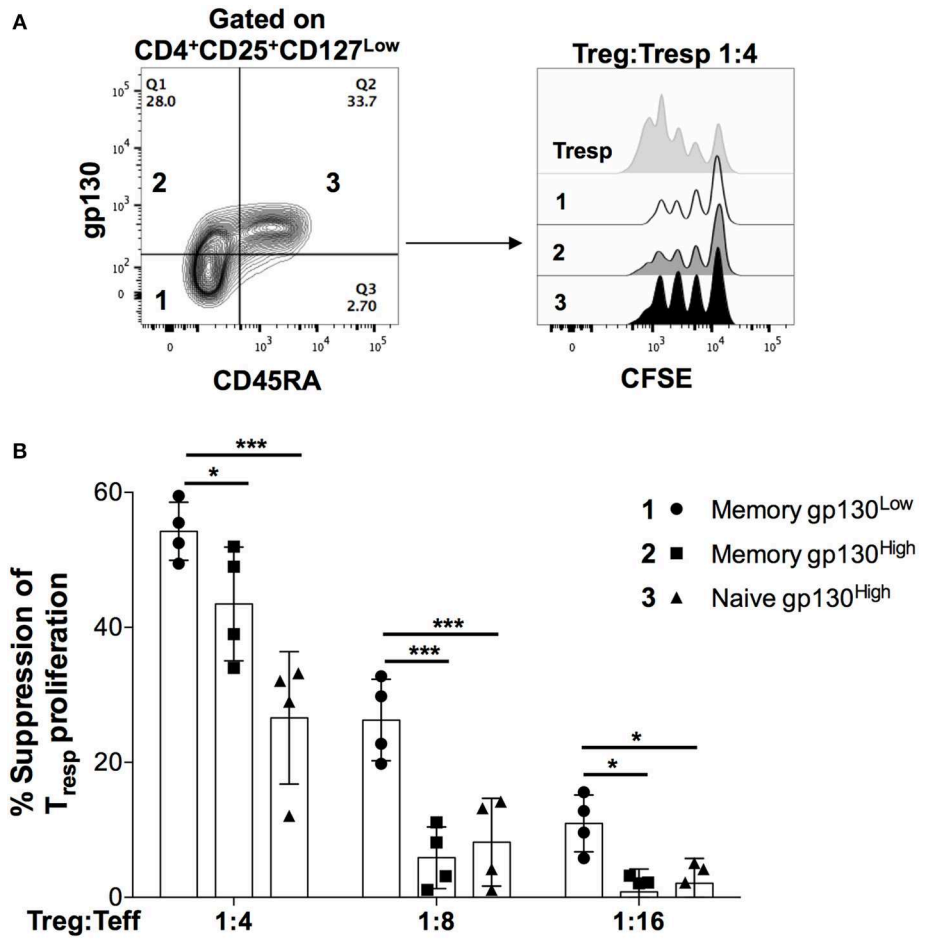

FIGURE 4 | High gp130 expression identifies Treg cells with reduced suppressive capacity *ex vivo*. Naïve and memory Treg cells ($CD4^+CD25^{High}CD127^{Low}$) cells were FACS-sorted according to their gp130 expression levels and co-cultured with CFSE-labeled, FACS-sorted $CD4^+CD25^-$ Teff cells in the presence of anti-CD3 and irradiated PBMCs for 96 h. Shown are representative FACS plots **(A)** and the percentage of suppression **(B)** of Teff cells at multiple Treg:Teff ratios in two different experiments on two different healthy donors. Statistical analysis was done with the one-way ANOVA followed by the Dunnett post-test. $^*p < 0.05$, $^{***}p < 0.001$.

measured their capacity to suppress the proliferation of TCR-activated $CD4^+CD25^-$ Teff cells in the presence of titrated amounts of IL-6 or IL-27. Following a 96-h incubation period, Teff cell proliferation was assessed as a measure of Treg cell suppressive capacity. Both IL-6 (**Figure 6A**) and IL-27 (**Figure 6B**) significantly reduced the suppressive function of Treg cells in a dose-dependent manner (**Figure 6A**). While IL-6 treatment also caused a significant increase in the proliferation of Teff cells in the absence of Treg cells, IL-27 did not directly alter Teff proliferation (**Figure 6C**). These data suggest that while both IL-6 and IL-27 inhibit the suppressive function of human Treg cells, IL-27 likely acts directly on Treg cells while IL-6 also indirectly impairs Treg function through enhancement of Teff cell proliferation.

Finally, we and others have previously described a key role for the Helios transcription in the functional stability of Treg cells (6, 43–45). We questioned whether IL-6 and IL-27-mediated loss of Treg suppressive function is associated with alterations in Helios expression. Indeed, we observed that both IL-6 and IL-27 cause a significant reduction in the levels of Helios expression in human Treg cells co-cultured with Teff cells (**Figure 6D**), suggesting that gp130 signaling, either directly or indirectly, could impact the regulatory network of Helios leading to impaired suppression by Treg cells. Further work is needed to elucidate the exact molecular mechanisms through which these gp130-signaling cytokines affect Helios expression and Treg function.

Blockade of the gp130 Receptor Augments Treg Cell Suppressive Activity

As both IL-6 and IL-27 signal through gp130 and abrogate Treg cell suppression, we next determined whether functional blockade of gp130 could rescue Treg cell suppressive activity. To achieve this, we made use of a recently described small molecule inhibitor of gp130, LMT-28, shown to potently inhibit gp130-mediated signaling (46). We first assessed the potency of the

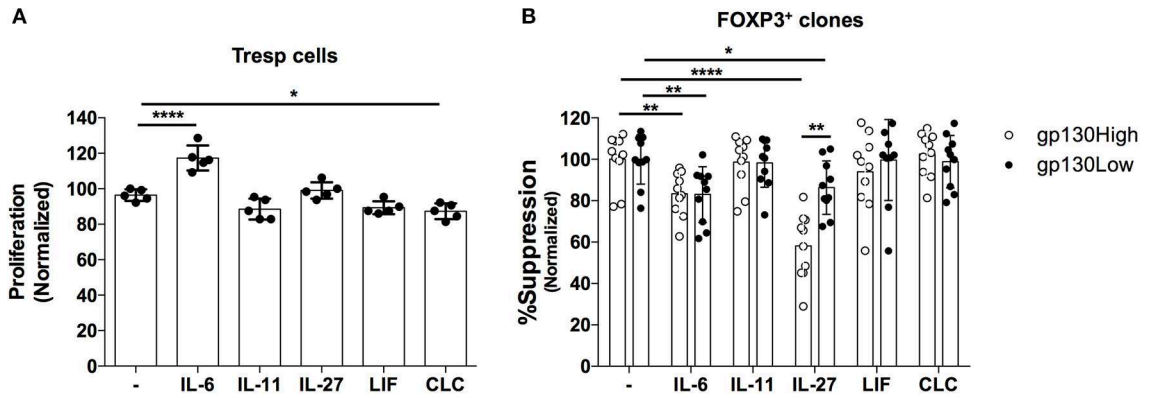

FIGURE 5 | Effect of gp130-binding cytokines on the suppressive activity of primary FOXP3$^+$ T cell clones. FOXP3$^+$ clones were generated from healthy donors and assessed for their ability to suppress allogeneic CD4$^+$CD25- Teff cells in a 1:1 Treg:Teff suppression assay in the absence of irradiated APCs. **(A)** The effects IL-6 (50 ng/mL), IL-11 (100 ng/mL), IL-27 (20 ng/mL), LIF (100 ng/mL), and CLC (1 ug/mL) on the proliferative response of Teff cells activated in the absence of clones. **(B)** The effects of the indicated cytokines on the suppressive potency of gp130^{High} (gp130 MFI > 600) vs. gp130^{Low} (gp130 MFI < 500) FOXP3$^+$ clones identified based on their gp130 expression levels before activation. Shown are the results from one representative experiment of three different experiments where clones were generated from three different healthy individuals. Statistical analysis was done with the one-way ANOVA followed by the Dunnett post-test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

gp130 inhibitor in preventing gp130-mediated cytokine signals in T cells by evaluating STAT3 phosphorylation.

IL-6-mediated signaling through gp130 activates STAT3, which is subsequently phosphorylated. In order to test the potency of the gp130 inhibitor, we evaluated STAT3 phosphorylation in the presence and absence of the drug in healthy human PBMCs stimulated with IL-6. Total healthy PBMCs were plated with the inhibitor for 1 h followed by stimulation with IL-6 for 10 min, after which STAT3 phosphorylation was evaluated using multi-parametric flow cytometry. We show that the gp130 inhibitor significantly abrogates IL-6-mediated STAT3 phosphorylation in CD4$^+$ T cells (**Figure 7A**).

Finally, we sought to determine whether gp130 inhibition could antagonize the effect of IL-6 signaling on the suppressive function of Treg cells. Indeed, while the suppressive function of naïve (CD45RA$^+$) Treg cells is significantly reduced by IL-6, treatment with the gp130 inhibitor is able to restore the suppressive capacity of Treg cells to normal levels (**Figure 7B**). These data further highlight the role of the gp130 axis in the regulation of Treg function, and demonstrate that gp130 may present a viable therapeutic target for the modulation of Treg activity.

DISCUSSION

We have previously reported on the remarkable degree of functional heterogeneity within human FOXP3$^+$ Treg cells. In particular, we showed that CD4$^+$CD25$^{High/Bright}$ Treg cells, albeit highly enriched in suppressive FOXP3$^+$ T cells, harbor a pool of *bona fide* FOXP3$^+$ Treg cells with compromised suppressive function, despite the maintenance of hallmark phenotypic and functional T_{reg} features. Notably, these non-suppressive Treg cells are indistinguishable from functionally suppressive Treg cells using the conventional markers of human Treg cells. In this study, we sought to identify potential factors that underlie such loss of function in FOXP3$^+$ cells. We demonstrated that APC-derived factors are responsible for the observed functional impairment, and identified gp130 as a surface receptor that is highly expressed in non-suppressive FOXP3$^+$ clones. We further showed that IL-6 and IL-27 negatively modulate Treg function. These results highlight the role of gp130 in regulating the function of human Treg cells and propose modulation of gp130 function as a potential therapeutic avenue for regulation of human Treg function in various disease settings.

Previous studies have associated IL-27 with both pro-inflammatory and anti-inflammatory roles in several animal models. As a pro-inflammatory cytokine, IL-27 signaling is a potent inducer of T-bet and IFN-γ (26–28), and plays a critical role in mediating CD4$^+$ T cell responses against chronic lymphocytic choriomeningitis virus (LCMV) infection (47). Moreover, in a model of helminth-induced inflammatory bowel disease (IBD), *Il27ra* deficiency impaired Th1 responses in the intestine resulting in inefficient worm expulsion and delayed onset of colitis (48). IL-27 has also been shown to inhibit the TGFβ-mediated induction of Treg cells (29), and adoptive transfer of *Il27ra*$^{-/-}$ Treg cells into lymphopenic mice resulted in attenuated colitis, which was attributed to increased induction of peripheral Treg cells (49). Furthermore, transgenic mice overexpressing IL-27 succumbed to spontaneous inflammation associated with a severe diminishment of their Treg pool (50). More recently, Zhu et al. reported that systemic delivery of IL-27 resulted in a rapid depletion of Treg cells and enhanced T cell-mediated inhibition of tumor growth in a melanoma mouse model (30). In contrast, a study by Do et al. reported that *Il27ra*$^{-/-}$ Treg cells are defective in their suppressive capacity and are unable to suppress inflammation

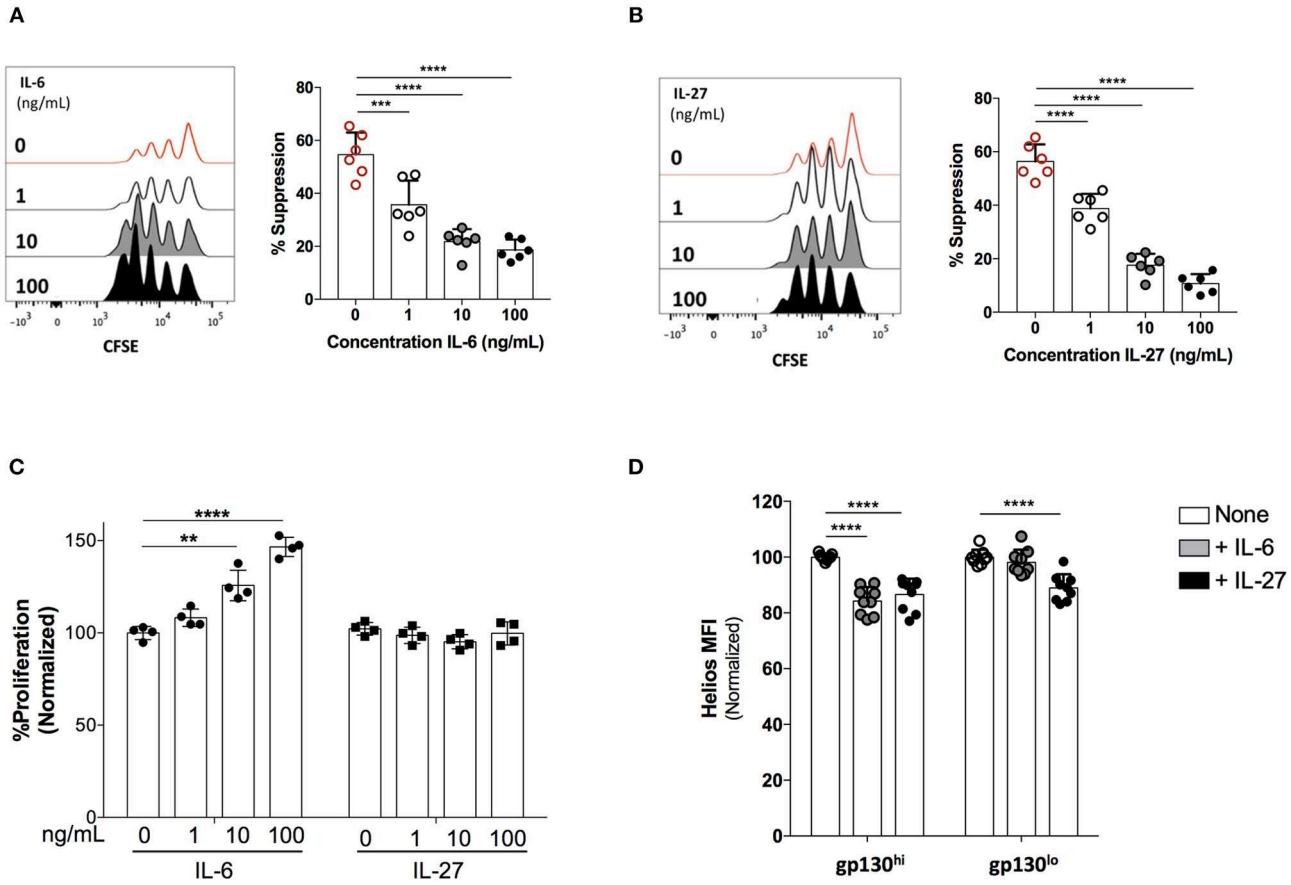

FIGURE 6 | IL-6 and IL-27 inhibit Treg cell suppressive function *ex vivo*. Treg cells ($CD4^+CD25^{High}CD127^{Low}$) cells were FACS-sorted and co-cultured with CFSE-labeled, FACS-sorted $CD4^+CD25^-$ Teff cells in the presence exogenous IL-6 or IL-27. Cells were stimulated with human αCD3/αCD28 coated beads (Treg Suppression Inspector; MACS Miltenyl Biotec) at a 1:2 bead: cell ratio for 96 h. Treg and Teff were plated at a ratio of 0:1 or 1:8, **(A)** The effects of rhIL-6 on the suppressive function of Treg cells. **(B)** The effects of rhIL-27 on the suppressive function of Treg cells. **(C)** The impact of IL-6 and IL-27 on the proliferative response of Teff cells activated in the absence of Treg cells. **(D)** $Gp130^{High}$ and $gp130^{Low}$ Treg cells ($CD4^+CD25^+CD127^{Low}$) were FACS-sorted and stimulated with human αCD3/αCD28 coated beads for 48 h in the presence of $CD4^+CD25^-$ Teff cells at a 1:4 Treg:Teff ratio. Where indicated, rIL-6 (10 ng/mL) or rIL-27 (10 ng/mL) was added. Helios expression was assessed by flow cytometry. Data are representative of at least three independent experiments performed on cells isolated from different healthy individuals. Statistical analysis was done with the one-way ANOVA followed by the Dunnett post-test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

in a colitis model (36). The authors further reported that stimulation of Treg cells in the presence of IL-27 substantially improved the suppressive function of Treg cells *in vitro* and *in vivo* (36). These seemingly contrasting results could be a result of the different mouse genetic backgrounds used in the aforementioned studies, C57BL/6 and BALB/c (30, 36, 49). Furthermore, it is becoming increasingly appreciated that differences in the composition of commensal microbiota have a significant influence on immune responses and disease outcome even within animals of the same strains (51), and therefore the influence of microbial composition in animal models on the these seemingly contrasting findings cannot be ruled out.

It should be noted that the majority of the studies that examined the *in vivo* effects of IL-27 have used $Il27ra^{-/-}$ mice. However, IL-27 may not be the only ligand for IL-27RA. Indeed, a recent study by Wang et al. has demonstrated that IL-35 also signals through a receptor complex involving the IL-27RA (52). IL-35 is an anti-inflammatory cytokine expressed by a suppressive subset of mouse T cells termed iTr35 (53, 54). IL-35 also shares the Ebi3 with IL-27 (53, 54). Thus, the shared nature of the IL-27RA complicates the interpretation of studies that relied on $Il27ra^{-/-}$ mice to examine the role of IL-27 *in vivo*.

Interestingly, Do et al. have also examined the effects of IL-27 on the suppressive function of human Treg cells and reported that IL-27 significantly improves their suppressive function (36). While this is in contrast to our findings showing a negative impact of IL-27 on human Treg function, there are important differences in the assessment of Treg function between the two studies. While we added IL-27 at the time of activation of the suppression co-culture, Do et al. activated Treg cells separately in the presence or absence of IL-27 for 3 days prior to co-culturing with Teff cells. Furthermore, Do et al. did not report details on the

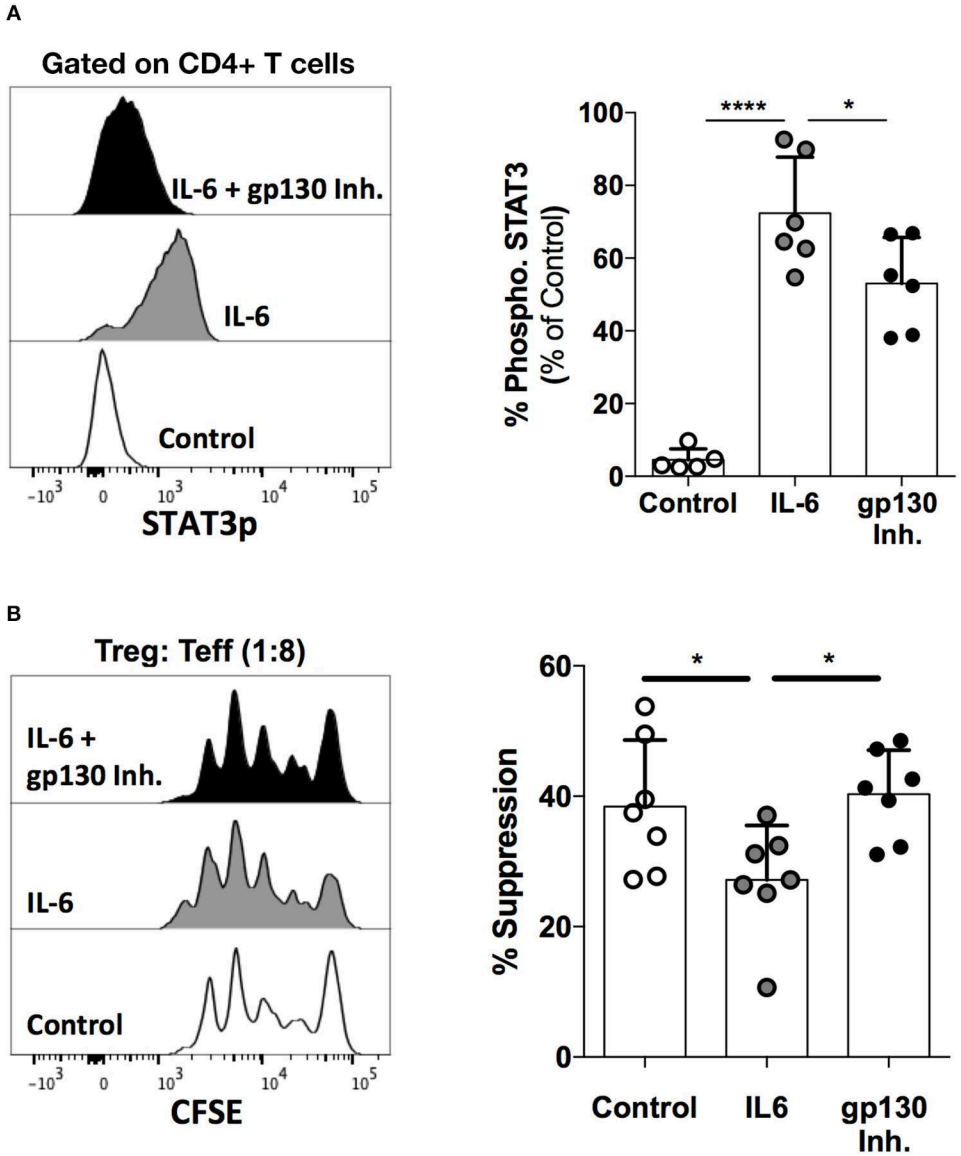

FIGURE 7 | Functional blockade of gp130 augments Treg cell function. **(A)** Healthy human PBMCs were incubated with the gp130 inhibitor LMT-28 (10 μM) for 1 h, followed by stimulation with rhIL-6 (10 ng/mL) for 10 min. STAT3p expression was evaluated using multi-parametric flow cytometry. Shown is STAT3 expression in gated $CD4^+$ T cells. **(B)** Naive Treg ($CD4^+CD45RA^+CD25^+CD127^{Low}$) cells were sorted from healthy human PBMCs and co-cultured with $CD25^-$ Teff cells in the presence of irradiated PBMCs and soluble anti-CD3 (30 ng/mL) for 96 h. Cells were plated at a 0:1 or 1:8 Treg: Teff cell ratio along with rhIL-6 (10 ng/mL) with or without LMT-28 (10 μM). Shown data is representative of 3 independent experiments. Statistical analysis was done with the one-way ANOVA followed by the Dunnett post-test. $^*p < 0.05$, $^{****}p < 0.0001$.

levels of activation achieved in their suppression assays to allow a clear estimation of the quality and magnitude of the modulation of Treg activity achieved by IL-27 (36). Importantly, we observed no effect of IL-27 on Teff cells cultured alone as a control, indicating that our observations of reduced Treg function in the presence of IL-27 are due to its direct action on Treg cells.

The mechanism through which IL-27 acts on Treg cells is unclear. However, previous studies have shown that IL-6 inhibits Treg function through the activation of the STAT3 pathway and overcoming the FOXP3-mediated inhibition of RORγt (55). IL-27 signaling through gp130 has also been shown to activate STAT3 (56, 57) and, therefore, it is possible that the mechanism of inhibition of Treg function is shared between IL-6 and IL-27. Additionally, IL-27 activates STAT1 leading to the inhibition of IL-2 production by T cells through the activation of the suppressor of cytokine signaling 3 (SOCS3) (50, 58, 59). Furthermore, IL-27 interferes with T cell responsiveness to IL-2 (30, 58). Given the vital role played by IL-2 in the survival and function of Treg cells, this latter effect of IL-27 likely plays a significant role in modulating Treg function.

An interesting observation in our study is the restoration of suppressive capacity in non-suppressive FOXP3$^+$ clones in the absence of APCs. This indicates that modulation of Treg function is largely mediated by local inflammatory factors, and is reversible, thus highlighting the resilient yet adaptable nature of human Treg cells in response to cues in the microenvironment. The significance of this functional heterogeneity is not clearly understood. One potential advantage of having subpopulations of Treg cells with differential responsiveness to inflammatory mediators would be the facilitation of a rapid down-modulation of Treg suppressive function in response to an infection. This would allow a partial relief of Treg activity and an enhanced initiation of an effective protective response. It would, therefore, be critical that down-modulated Treg cells can regain their suppressive capacity upon pathogen clearance. Whether deregulated and chronic production of inflammatory mediators can cause permanent loss of Treg function and contribute to autoimmunity remains to be investigated. There is, however, mounting evidence in mouse models indicating that Treg cells can, under inflammatory conditions such as lymphopenia or chronic infections, lose their Foxp3 expression and suppressive function and differentiate into Teff-like cells with inflammatory potential (60–62). It is not clear if these former Foxp3$^+$ cells can regain Foxp3 expression and suppressive capacity in homeostatic conditions *in vivo*. Persistent FOXP3 loss in a highly inflammatory environment may cause long-lasting quantitative and qualitative defects in the Treg population.

Our study also highlights the importance of considering the influence of the inflammatory milieu on Treg cells when assessing Treg function in human autoimmune disease. Although intrinsic defects in Treg function may be a possible underlying cause of organ-specific autoimmunity, it is highly likely that Treg dysfunction could be a consequence of functional modulation by extrinsic factors that are abundant *in situ* in inflammatory conditions. Monitoring Treg cells with an increased susceptibility to functional modulation, gp130^{high} cells for instance, in autoimmune patients could provide valuable insight into the functional status of the Treg population in these patients. Regardless of whether Treg dysfunction is causative or secondary in organ-specific autoimmunity, identification of pathways that interfere with Treg function is highly needed in order to design strategies through which Treg function can either be enhanced to allow a better control of autoimmune responses, or abrogated to bolster anti-tumor responses.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65650; https://www.ncbi.nlm.nih.gov/pubmed/25762785/.

ETHICS STATEMENT

Blood was obtained from healthy volunteers after informed consent. All samples used in this study were collected in accordance with the ethical review board of the Research Institute of the McGill University Health Center.

AUTHOR CONTRIBUTIONS

KB, ED'H, and CP designed the project. KB, SB, and ED'H performed the experiments and analyzed the data. KB and CP wrote the manuscript.

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The Modulation of Regulatory T Cells via HMGB1/PTEN/β-Catenin Axis in LPS Induced Acute Lung Injury

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Sepsis-induced acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) remains the leading complication for mortality caused by bacterial infection. The regulatory T (Treg) cells appear to be an important modulator in resolving lung injury. Despite the extensive studies, little is known about the role of macrophage HMGB1/PTEN/β-catenin signaling in Treg development during ALI.

Objectives: This study was designed to determine the roles and molecular mechanisms of HMGB1/PTEN/β-catenin signaling in mediating $CD4^+CD25^+Foxp3^+$ Treg development in sepsis-induced lung injury in mice.

Setting: University laboratory research of First Affiliated Hospital of Anhui Medical University.

Subjects: PTEN/β-catenin Loxp and myeloid-specific knockout mice.

Interventions: Groups of $PTEN^{loxp}$/β-$catenin^{loxp}$ and myeloid-specific PTEN/β-catenin knockout ($PTEN^{M-KO}$/β-$catenin^{M-KO}$) mice were treated with LPS or recombinant HMGB1 (rHMGB1) to induce ALI. The effects of HMGB1-PTEN axis were further analyzed by *in vitro* co-cultures.

Measures and Main Results: In a mouse model of ALI, blocking HMGB1 or myeloid-specific PTEN knockout ($PTEN^{M-KO}$) increased animal survival/body weight, reduced lung damage, increased TGF-β production, inhibited the expression of RORγt and IL-17, while promoting β-catenin signaling and increasing $CD4^+CD25^+Foxp3^+$ Tregs in LPS- or rHMGB-induced lung injury. Notably, myeloid-specific β-catenin ablation (β-$catenin^{M-KO}$) resulted in reduced animal survival and increased lung injury, accompanied by reduced $CD4^+CD25^+Foxp3^+$ Tregs in rHMGB-induced ALI. Furthermore, disruption of macrophage HMGB1/PTEN or activation of β-catenin significantly increased $CD4^+CD25^+Foxp3^+$ Tregs *in vitro*.

Conclusions: HMGB1/PTEN/β-catenin signaling is a novel pathway that regulates Treg development and provides a potential therapeutic target in sepsis-induced lung injury.

Keywords: acute lung injury, HMGB1, regulatory T cells, sepsis, inflammation

INTRODUCTION

Sepsis is a systemic inflammatory response syndrome which may result in acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS) (1). ARDS is a type of respiratory failure characterized by rapid onset of widespread inflammation in the lungs, symptoms include shortness of breath, rapid breathing, and bluish skin coloration (2). Despite recent progress in developing many pharmacological interventions for ALI/ARDS, there have been no successful clinical trials for drugs treating these disorders, implying that there are complex molecular mechanisms in sepsis-driven inflammatory responses.

High-mobility group box 1 protein (HMGB1), a highly conserved and ubiquitous DNA binding nuclear protein, is a key mediator during inflammatory responses in sepsis (3). HMGB1, as an innate "danger signal" (alarmin), plays a key role in the initiating innate and adaptive immune response (4–6). As a late mediator, HMGB1 can be actively released from endotoxin-stimulated macrophages following lipopolysaccharide (LPS) and by TNF-α or IL-1β stimulation. Blockade of HMGB1 via antibody targeting protects against LPS lethality in mice, whereas administration of HMGB1 in mice results in developing endotoxemia and lethality (7). HMGB1 contributes to the endotoxin-induced ALI through activating NF-κB translocation, increasing levels of proinflammatory cytokines, and enhancing lung permeability (8–10). Extracellular HMGB1 augmented autoimmune response through stimulating dendritic cell maturation and macrophage activation, whereas HMGB1 deficiency resulted in increasing the number of lymph node $CD4^+Foxp3^+$ regulatory T (Treg) cells during inflammatory response (11). Moreover, disruption of HMGB1 promotes the ability to induce Treg and enhances antitumor immunity (12).

Recently, $CD4^+CD25^+Foxp3^+$ Tregs have been shown to be crucial for the resolution of endotoxin-induced lung injury via both TGF-β-dependent and -independent pathways (13). TGF-β induces Treg-mediated suppressive activity and Foxp3 expression (14, 15). The development and survival of $CD4^+CD25^+$ Tregs *in vivo* was depressed by the increased phosphatase and tensin homolog deleted on chromosome ten (PTEN) activity via distinct IL-2 receptor (IL-2R) signaling, which is associated with downstream mediators of PI3K (16). Deficiency of myeloid PTEN increases PI3K signaling and reduces endotoxin-induced inflammatory response and lung injury (17). Indeed, loss of PTEN leads to an increasing nuclear accumulation of β-catenin (18) and promotes PI3K, which P3 and activates downstream PDK1 and Akt (19). Increasing phosphorylation of Akt by PDK1 enhances Akt activity and facilitates Treg induction (20), whereas deletion of PDK1 in T cells results in reducing Treg numbers *in vitro* and *in vivo* (21). Thus, the modulation of Treg development might involve in multiple pathways during lung inflammation and injury.

Using a well-established model of lung injury and an *in vitro* co-culture system, we identified a novel regulatory pathway of HMGB1/PTEN/β-catenin signaling on Treg induction during inflammatory response. We demonstrated that HMGB1 promoted lung inflammation through activating myeloid PTEN-mediated innate immunity. Lacking myeloid PTEN ultimately resulted in promoting β-catenin activation and TGF-β production, which in turn induced $CD4^+CD25^+Foxp3^+$ Tregs and suppressed endotoxin-mediated inflammation in the lung. Our data document that HMGB1/PTEN/β-catenin signaling is critical for development of Tregs in the resolution of sepsis-induced lung injury.

MATERIALS AND METHODS

Mice

The floxed β-catenin ($\beta\text{-catenin}^{flox}$) mice (The Jackson Laboratory, Bar Harbor, ME), and the mice expressing Cre recombinase under the control of the Lysozyme M (LysM) promoter (LysM-Cre; The Jackson Laboratory) were used to generate myeloid-specific β-catenin knockout ($\beta\text{-catenin}^{M-KO}$) mice. In brief, homozygous $\beta\text{-catenin}^{flox}$ mice were interbred with homozygous LysM-Cre mice, and the heterozygous offspring were then backcrossed to the homozygous $\beta\text{-catenin}^{flox}$ mice to generate $\beta\text{-catenin}^{M-KO}$ (LysM-Cre-$\beta\text{-catenin}^{flox}$) mice. The C57BL/6 wild-type (WT) and $PTEN^{flox}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The expression of β-catenin was detected in spleen and myloid cells, respectively (**Figure S1**). The myeloid-specific PTEN knockout ($PTEN^{M-KO}$) mice were generated as described (22). Mouse genotyping was performed by using a standard protocol with primers described in the JAX Genotyping protocols database, and the expression of PTEN was detected as described (22). All animals were housed in animal facility under specific pathogen-free conditions. Animals at 8–10 weeks of age were used in all experiments.

Mice Treatment

To establish the animal model of ALI, mice were anesthetized with i.p. ketamine (150 mg/kg) and acetylpromazine (13.5 mg/kg), and then an incision (1–2 cm) was made on the animal neck to expose the trachea. A 20-gauge catheter was inserted into the lumen of trachea. 50 μl of LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, 100 μg/mouse), diluted in sterile water was instilled via the catheter. Sterile water was used in the control group (8–10 mice per group) (13). To determine the role of HMGB1 during LPS-induced ALI, mice were instilled with 100 μg/mouse of anti-HMGB1 (Product# 326052233, Shino-TEST Co, Tokyo, Japan) immediately after LPS instillation. Control mice received the same volume of saline solution or control IgG (Sigma-Aldrich). To generate mouse model of endotoxin-induced sepsis, mice were injected with LPS (750 μg/mouse, i.p.) as described (23). In some experiments, mice were administrated with recombinant HMGB1 (rHMGB1, 50 μg/mouse, i.p., product# 4652, Sigma-Aldrich) or vehicle PBS. Since previous reports showed that maximal lung injury and HMGB1 expression occurred between 12 and 48 h after LPS instillation (24), all animal studies were executed at 24 h after LPS, rHMGB1, anti-HMGB1, control IgG or saline treatment.

Analysis of the Permeability Index

The permeability index, reflexing the damage of alveolar epithelial and endothelial permeability, was evaluated by

administrating human serum albumin (i.v. 25 μg; Signa-Aldrich, MO) 1 h prior to sacrificing the animal. The blood and BALF were collected at the time of sacrifice. ELISA assay was performed to measure the level of human albumin concentration using a human serum albumin ELISA kit (Cayman Chemical, Ann Arbor, MI). The pulmonary permeability index was defined as the human albumin concentration in BAL fluid/serum ratio.

Analysis of Bronchoalveolar Lavage Fluid (BALF)

The mice were anesthetized before exposure of the trachea. After the catheter was inserted into the lumen of trachea, the lungs were then lavaged 3 times with 0.8 ml of sterile saline. The total collected lavage averaged 1.4–1.7 ml/mouse. BALF was centrifuged at 800 × g for 10 min at 4°C. The cell-free supernatants were stored at −80°C for later analysis. The cell pellet was re-suspended in PBS and counted by a hemacytometer. The differential staining was performed with Diff-Quik staining solutions to count enriched alveolar macrophages as described (25).

Analysis of HMGB1 and Cytokines

The mouse ELISA kits were used to measure the levels of HMGB1 (Shino-TEST Co, Tokyo, Japan), TGF-β, TNF-α, IL-1β, IL-17A, and IL-23 (p19) (eBioscience) in BALF, serum and co-cultures according to the manufacturer's instructions.

Histological Analysis

The lungs from mice ($n = 8$/group) were harvested and rinsed with PBS, and then immersed into 10% of buffered formalin overnight. After processing for paraffin embedding, the lung sections were stained with hematoxylin and eosin (H&E). The severity of lung injury was evaluated semi-quantitatively by grading score on a scale from 1 to 5 as described (13). In this classification, 1, normal; 2, focal (<50% lung section) interstitial congestion and inflammatory cell infiltration; 3, diffuse (>50%

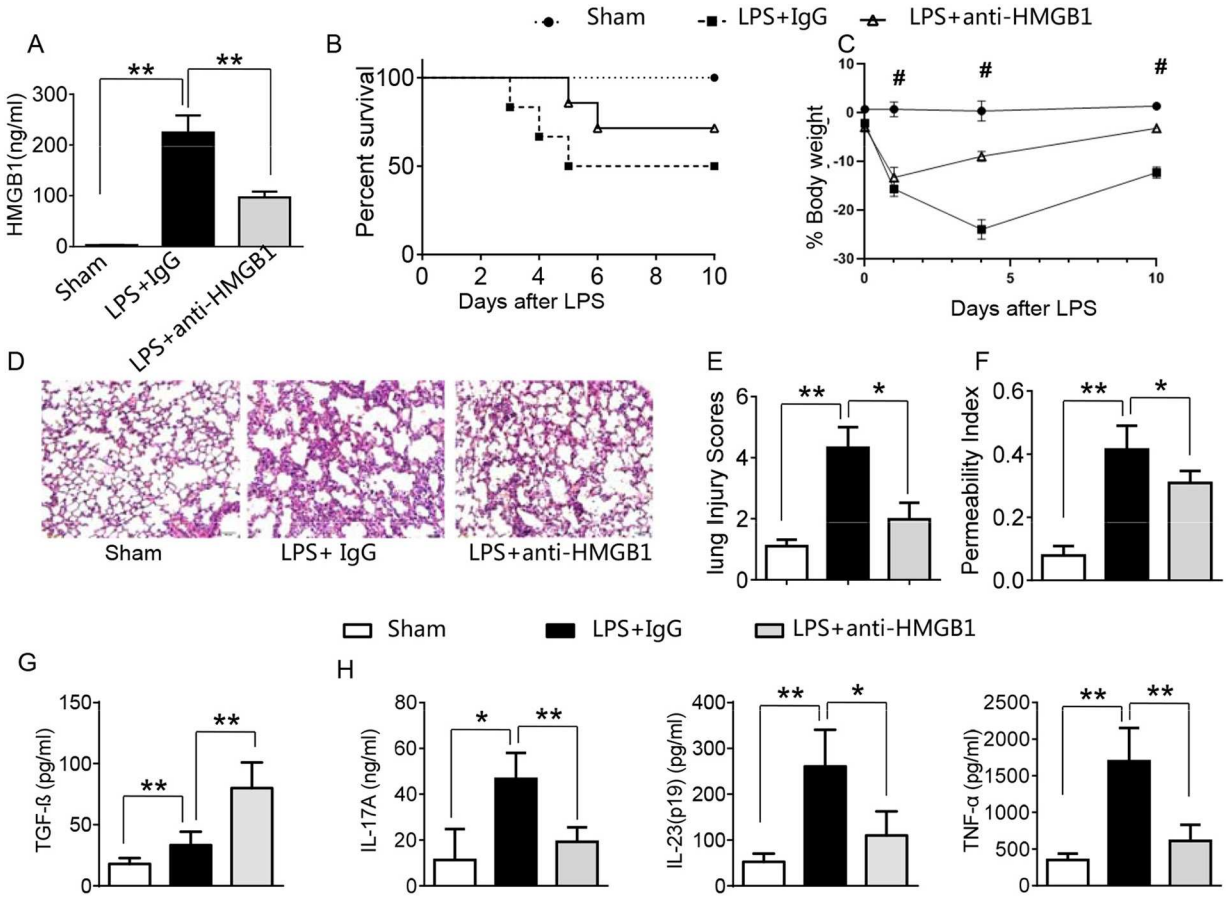

FIGURE 1 | Blocking HMGB1 ameliorates lung damage, increases TGF-β production, and suppresses proinflammatory mediators in acute lung injury. Mice were subjected to LPS or anti-HMGB1 instillation via a catheter after exposure of the trachea. **(A)** HMGB1 levels were measured by ELISA assay in BAL fluid from sham, LPS-stilled, and anti-HMGB1 antibody-treated mice. Mean±SD ($n = 4$–6 samples/group), $^{**}p < 0.01$. **(B)** Animal survival ($n = 8$ animals per group). **(C)** Body weight ($n = 8$). **(D)** Lung sections were stained with H&E. Original magnification, ×40; **(E)** Histopathological mean lung injury scores ($n = 8$ animals per group); $^{**}p < 0.01$. **(F)** The pulmonary permeability index was measured in sham, LPS-instilled, and anti-HMGB1 antibody-treated mice ($n = 8$ animals per group); $^{**}p < 0.01$. ELISA assay was performed in BAL fluid from sham, LPS-stilled, and anti-HMGB1 antibody-treated mice **(G)** TGF-β, **(H)** IL-17A, IL-23 (p-19), and TNF-α; Mean ± SD ($n = 4$-6 samples/group), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$ between LPS + IgG and LPS+anti-HMGB1 group.

lung section) interstitial congestion and inflammatory cell infiltration; 4, focal (<50% lung section) consolidation and inflammatory cell infiltration; 5, focal (>50% lung section) consolidation and inflammatory cell infiltration. The mean score was determined by examining each sample.

Myeloperoxidase Activity Assay

The presence of myeloperoxidase (MPO) was used as an index of lung neutrophil accumulation as described (26). The frozen tissue samples were homogenized and separated by centrifugation. Supernatants were analyzed for MPO activity by spectrophotometry at 655 nm, and the change in absorbance was measured. One unit of MPO activity was defined as the quantity of enzyme degrading 1 μmol peroxide/min at 25°C per gram of tissue.

Western Blot Analysis

Protein was extracted from macrophages with ice-cold protein lysis buffer (50 mM Tris, 150 mM Nacl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton-100). The buffer contains 1% proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich). Proteins (30 μg/sample) in SDS-loading buffer (50 mM Tris, pH 7.6, 10% glycerol, 1% SDS) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% dry milk and 0.1% Tween 20 (USB, Cleveland, OH). Monoclonal rabbit anti-mouse HMGB1 (product# 6893), PTEN (product# 9188), β-catenin (product# 8480), phos-PDK1 (product# 3438), phos-Akt (ser473) (product# 4060), and β-actin (product# 3700) Abs (Cell Signaling Technology, MA) were used. The membranes were incubated with Abs, and then developed according to the Pierce SuperSignal West Pico Chemiluminescent Substrate protocol (Pierce Biotechnology, Rockford, IL). Relative quantities of protein were determined and expressed in absorbance units (AU) comparing to β-actin expression using a densitometer (Kodak Digital Science 1D Analysis Soft-ware, Rochester, NY).

Quantitative RT-PCR Analysis

Total RNA was purified from lung tissue, peripheral blood or spleen T cells using RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Reverse

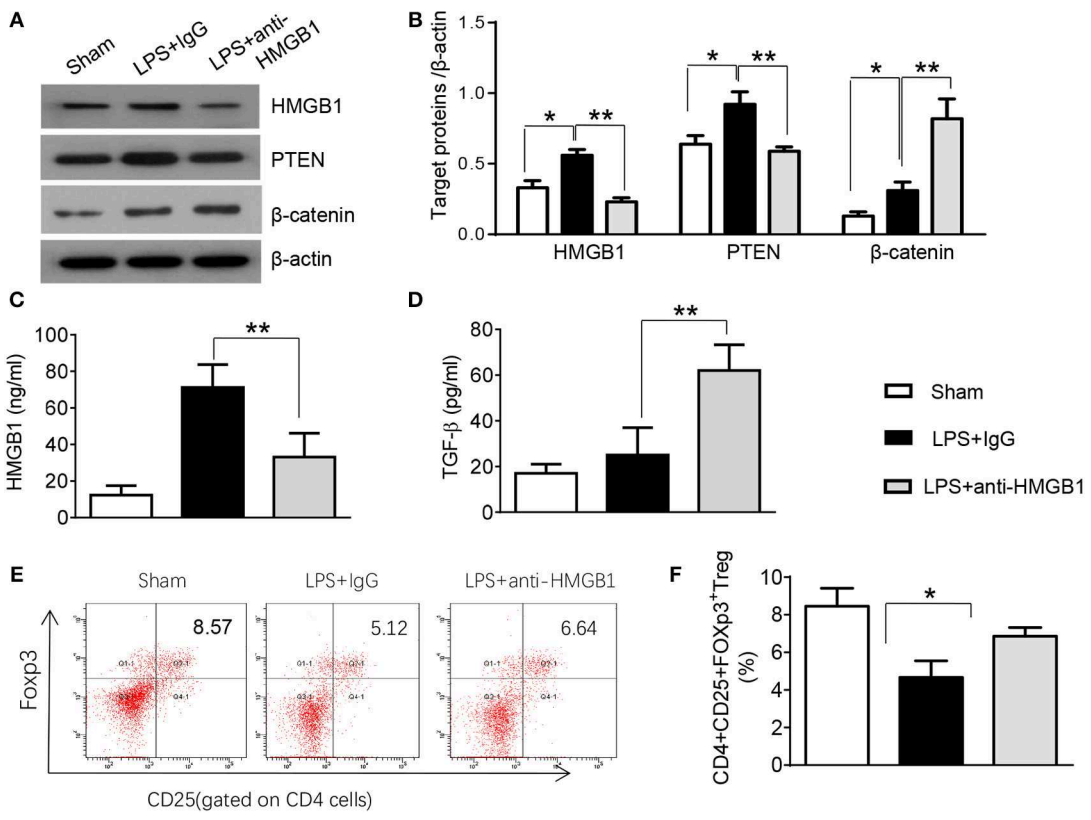

FIGURE 2 | Blocking HMGB1 inhibits PTEN but promotes β-catenin activation and Tregs in acute lung injury. **(A)** The protein was isolated from alveolar macrophages in BAL fluid from sham, LPS-instilled, and anti-HMGB1 antibody-instilled mice. The expression of HMGB1, PTEN, and β-catenin was analyzed by Western blots. Representative of three experiments. **(B)** The density ratio of HMGB1, PTEN, and β-catenin. *$p < 0.05$. ELISA assay was performed in serum from sham, LPS-instilled, and anti-HMGB1 antibody-instilled mice **(C)** HMGB1, **(D)** TGF-β, Mean ± SD ($n = 4$–6 samples/group), **$p < 0.01$. **(E)** Representative three-dimension scatter diagrams of $CD4^+CD25^+Foxp3^+$ Tregs in the peripheral blood from sham, LPS-instilled, and anti-HMGB1 antibody-instilled mice were analyzed by flow cytometry. **(F)** The percentage of $CD4^+CD25^+Foxp3^+$ Tregs in the peripheral blood from sham, LPS-instilled, and anti-HMGB1 antibody-instilled mice ($n = 4$–6 animals/group), Mean ± SD, *$p < 0.05$.

transcription to cDNA was performed by using SuperScript III First Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25 μl, the following were added: 1 × SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen, San Diego, CA) cDNA and 10 μM of each primer. Amplification conditions were: 50°C (2 min), 95°C (5 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s). Primer sequences used for the amplification of TNF-α, TGF-β, IL-17A, IL-23, RORγt, Foxp3, and HPRT are shown in **Supplementary Table 1**. Target gene expressions were calculated by their ratios to the housekeeping gene HPRT.

Cell Isolation

The WT, PTEN^{flox}, PTEN^{M-KO}, β-catenin^{flox}, and β-catenin^{M-KO} mice were anesthetized with sodium pentobarbital (100 mg/kg, i.p.), and then Bio-Gel elicited peritoneal macrophages were isolated as described previously (22). The macrophages were cultured in medium (Invitrogen) supplemented with 10% FBS, 100 μg/ml of penicillin/streptomycin (Life Technologies; Grand Island, NY). The peripheral blood or spleen T cells were purified using the EasySep^{TM} mouse T cell isolation kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. T cells were then stimulated with anti-CD3 (1 μg/ml, Clone 145-2C11) and anti-CD28 (2 μg/ml, Clone 37.51) (eBioscience).

In vitro Transfection and Treatments

After 24 h cell culture, 1 × 10^6 macrophages/well were transfected with 100 nM of HMGB1 siRNA or non-specific control siRNA using lipofectamine 2000 reagent (Invitrogen), and incubated for 24 h. Non-specific (NS) siRNA as a control. In some experiments, cells were pretreated with 10 μg/ml of

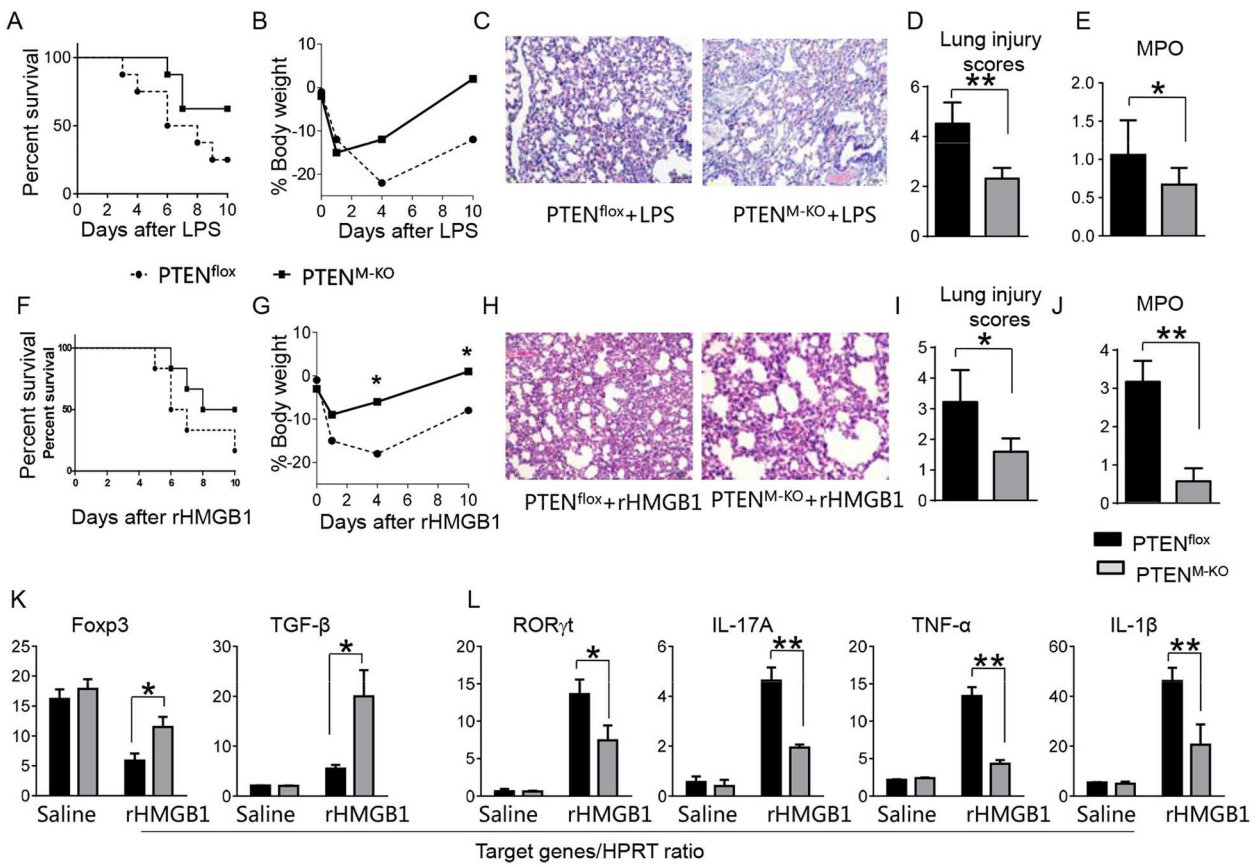

FIGURE 3 | Myeloid cell-specific PTEN is critical for the HMGB1-mediated inflammatory response in acute lung injury. PTEN^{flox} and PTEN^{M-KO} Mice were given LPS or rHMGB1 at 24 h prior to lung tissue, blood, and cell harvest. **(A)** Animal survival after LPS treatment ($n = 8$ animals per group). **(B)** Body weight after LPS treatment ($n = 8$). **(C)** Lung sections from PTEN^{flox} and PTEN^{M-KO} mice after LPS treatment were stained with H&E. Original magnification, ×40; **(D)** Histopathological mean lung injury scores ($n = 8$); $^{**}p < 0.01$. **(E)** MPO assay in PTEN^{flox} and PTEN^{M-KO} mice after LPS treatment ($n = 8$ samples/group), $^{**}p < 0.01$. **(F)** Animal survival after rHMGB1 treatment ($n = 8$ animals per group). **(G)** Body weight after rHMGB1 treatment ($n = 8$). **(H)** Lung sections from PTEN^{flox} and PTEN^{M-KO} mice after rHMGB1 treatment were stained with H&E. Original magnification, ×40; **(I)** Histopathological mean lung injury scores ($n = 8$ animals/group); $^{*}p < 0.05$. **(J)** MPO assay in PTEN^{flox} and PTEN^{M-KO} mice after rHMGB1 treatment ($n = 8$ samples/group), $^{**}p < 0.01$. q-PCR analysis of mRNA expression coding for **(K)** Foxp3 and TGF-β, **(L)** RORγt, IL-17A, TNF-α, and IL-1β in lung tissues from PTEN^{flox} and PTEN^{M-KO} mice after rHMGB1 treatment. Mean ± SD ($n = 4$–6 samples/group), $^{**}p < 0.01$. Representative mean values of cytokine gene mRNA copies normalized to HPRT control.

rHMGB1 or 10 μg/ml of anti-HMGB1 for 24 h, and then were supplemented with 1 μg/ml of LPS for additional 6 h. The HMGB1 siRNA and control siRNA were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Macrophage/T Cell Co-cultures

The HMGB1 siRNA-transfected macrophages or macrophages isolated from WT, $PTEN^{flox}$, $PTEN^{M-KO}$, β-$catenin^{flox}$, and β-$catenin^{M-KO}$ mice were suspended at 5×10^5 cells/ml and cultured on 60 mm plates. After the cells were stimulated with LPS (1 μg/ml) for 6 h, spleen T cells were then added into cultures at a macrophage/T cell ratio of 1:10 as described before (27). The co-cultured cells were incubated for 24 h, and then macrophages and spleen T cells were harvested for the Western blots, real-time PCR, and flow cytometry analysis.

Flow Cytometry Analysis

Peripheral blood T cells isolated from LPS- and/or anti-HMGB1-treated WT, or rHMGB1-treated $PTEN^{flox}$, $PTEN^{M-KO}$, β-$catenin^{flox}$, and β-$catenin^{M-KO}$ mice, as well as spleen T cells harvested from co-cultures were stained with anti-mouse CD4-PE-Cyanine5 (RM4-5), CD25-PE (PC61.5), and Foxp3-FITC (FJK-16s) mAbs (eBioscience) according to the manufacturer's instructions. PE-labeled rat anti-mouse IgG2a isotypes were used as negative controls. Measurements were performed using a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using CellQuest software.

Statistical Analysis

All experiments were repeated three times. Data are expressed as mean±SD and analyzed by Permutation *t*-test and Pearson

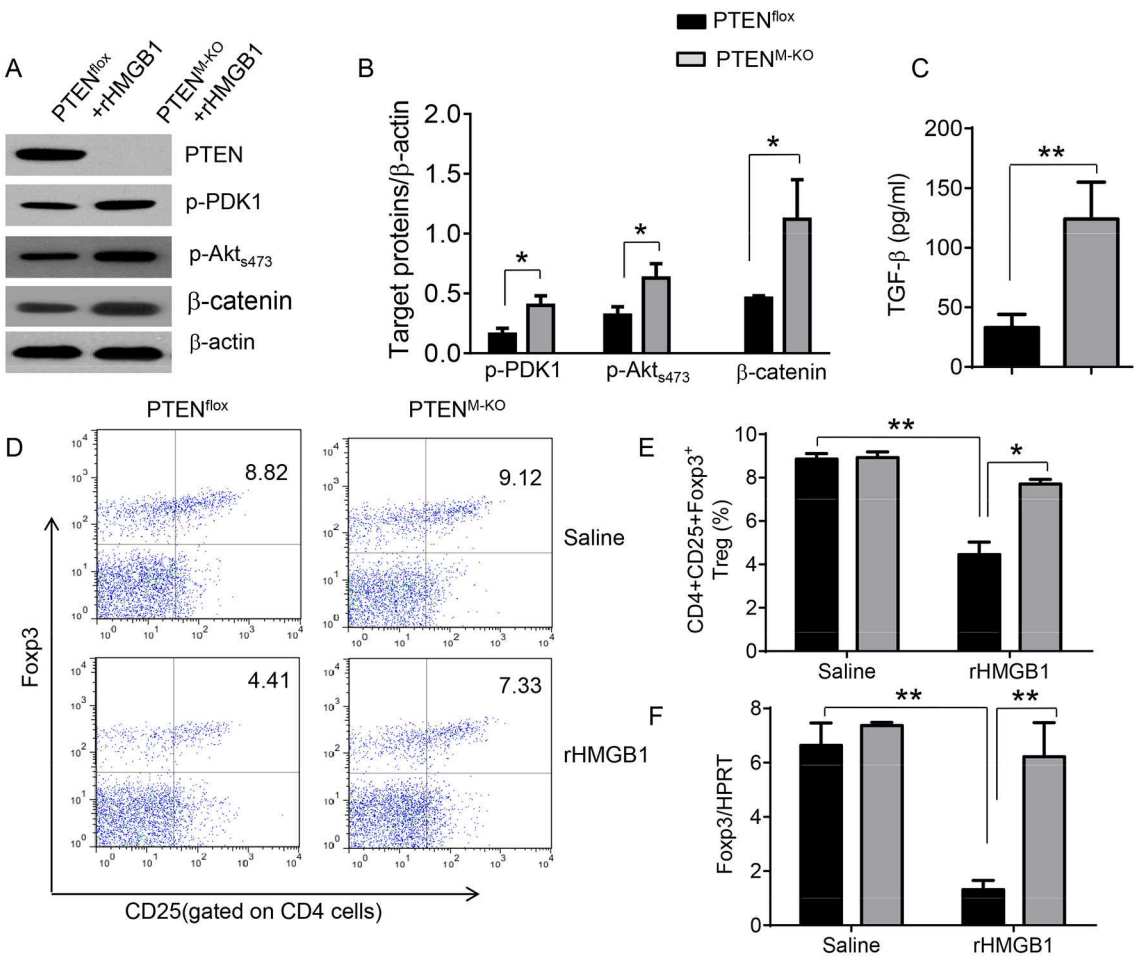

FIGURE 4 | Myeloid cell-specific PTEN deficiency is critical for the induction of Tregs in acute lung injury. Mice were given rHMGB1 at 24 h prior to lung tissue, blood, and cell harvest. **(A)** The protein was isolated from peritoneal macrophages in $PTEN^{flox}$ and $PTEN^{M-KO}$ mice after rHMGB1 treatment. The expression of PTEN, p-PDK1, p-Akt (ser473), and β-catenin was analyzed by Western blots. Representative of three experiments. **(B)** The density ratio of p-PDK1, and p-Akt (ser473), and β-catenin. $^*p < 0.05$, $^{**}p < 0.01$. **(C)** ELISA-based detection of TGF-β levels in serum from $PTEN^{flox}$ and $PTEN^{M-KO}$ mice after rHMGB1 treatment. Mean ± SD (n = 4–6 samples/group), $^{**}p < 0.01$. **(D)** Representative diagrams of $CD4^+CD25^+Foxp3^+$ Tregs in the peripheral blood from $PTEN^{flox}$ and $PTEN^{M-KO}$ mice after rHMGB1 treatment was analyzed by flow cytometry. **(E)** The percentage of $CD4^+CD25^+Foxp3^+$ Tregs in the peripheral blood from $PTEN^{flox}$ and $PTEN^{M-KO}$ mice after rHMGB1 treatment (n = 4–6 animals/group), Mean±SD, $^*p < 0.05$. **(F)** q-PCR analysis of mRNA expression coding for Foxp3 in peripheral blood T cells from $PTEN^{flox}$ and $PTEN^{M-KO}$ mice after rHMGB1 treatment. Mean ± SD (n = 4–6 samples/group), $^{**}p < 0.01$.

correlation. Per comparison two-sided p-values <0.05 were considered statistically significant. Multiple group comparisons were performed using one-way ANOVA with the *post-hoc* test. The body weight loss was analyzed by using student's t-test. All analyses were made using SAS/STAT software, version 9.4.

RESULTS

Blocking HMGB1 Ameliorates Lung Damage, Increases TGF-β Production, and Suppresses Proinflammatory Mediators in Acute Lung Injury

LPS has been shown to induce HMGB1 release and triggers systemic inflammatory response in sepsis (3, 13, 23). Using the mouse model of LPS-induced ALI, we found that instillation of LPS significantly increased HMGB1 levels in BALF compared to sham controls (**Figure 1A**, 224.6 ± 33.7 vs. 2.6 ± 0.33, $p < 0.01$). In contrast, neutralized HMGB1 release with polyclonal anti-HMGB1 treatment reduced HMGB1 levels (96.9 ± 11.5, $p < 0.01$). Furthermore, unlike in IgG controls, anti-HMGB1 treatment increased animal survival (**Figure 1B**, 67.3 vs. 48.5%, $p < 0.05$) at day 6. The surviving anti-HMGB1-treated mice continued to appear gained weight from days 4–10 (**Figure 1C**, −11.9 to −3.7%, $p < 0.05$) compared to IgG controls (−22.7 to −13.6%). Indeed, instillation of anti-HMGB1 showed less interstitial congestion, inflammatory cell infiltration and proteinous exudate into the alveoli, compared to mice that received control IgG (**Figures 1D,E**, 1.7 ± 0.55 vs. 4.3 ± 0.68, $p < 0.01$). The lung permeability index (LPI) was significantly decreased in anti-HMGB1 group compared to IgG controls after LPS instillation (**Figure 1F**, 0.31 ± 0.04 vs. 0.42 ± 0.08, $p < 0.05$). As TGF-β might play an important role in the resolution of lung injury (28), we also measured its levels in BALF. We found instillation of anti-HMGB1 significantly increased TGF-β levels,

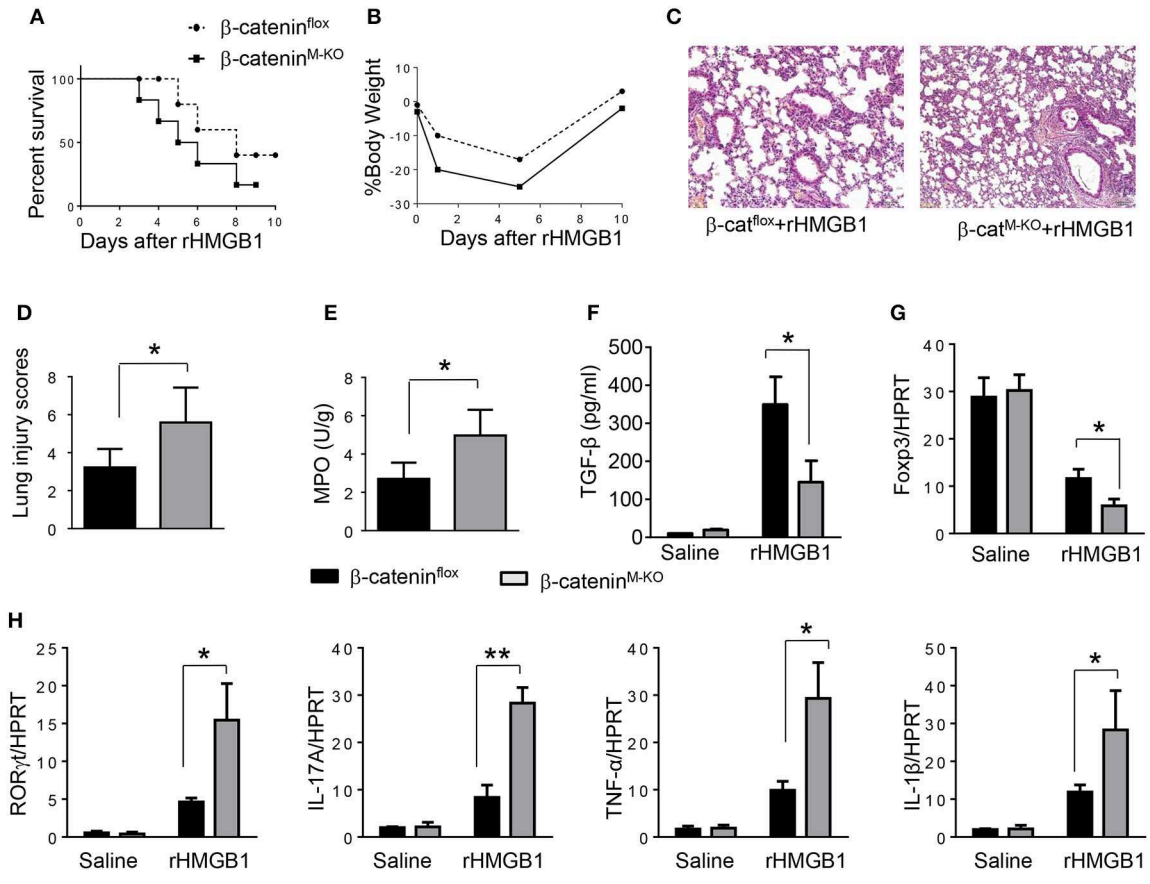

FIGURE 5 | Myeloid β-catenin signaling is essential for the induction of $CD4^+CD25^+Foxp3^+$ Treg in lung injury. The β-catenin^{flox} and β-catenin^{M-KO} mice were given rHMGB1 at 24 h prior to lung tissue and blood harvest. **(A)** Animal survival after rHMGB1 treatment ($n = 8$ animals per group). **(B)** Body weight after rHMGB1 treatment ($n = 8$). **(C)** Lung sections from β-catenin^{flox} and β-catenin^{M-KO} mice after rHMGB1 treatment were stained with H&E. Original magnification, ×40. **(D)** Histopathological mean lung injury scores ($n = 8$); $^{**}p < 0.01$. **(E)** MPO assay in β-catenin^{flox} and β-catenin^{M-KO} mice after rHMGB1 treatment ($n = 8$ samples/group). **(F)** ELISA-based detection of TGF-β in serum from β-catenin^{flox} and β-catenin^{M-KO} mice after rHMGB1 treatment. Mean±SD ($n = 4$–6 samples/group). q-PCR analysis of mRNA expression coding for **(G)** Foxp3 and **(H)** RORγt, IL-17A, TNF-α, and IL-1β in lung tissues from β-catenin^{flox} and β-catenin^{M-KO} mice after rHMGB1 treatment. Mean ± SD ($n = 4$–6 samples/group). Representative mean values of cytokine gene mRNA copies normalized to HPRT control. $^*p < 0.05$, $^{**}p < 0.01$.

as compared with controls (**Figure 1G**, 80.35 ± 21.74 vs. 33.3 ± 10.97, $p < 0.01$). Indeed, IgG-treated controls showed elevated levels of IL-17A, IL-23 (p19), and TNF-α in BALF (**Figure 1H**), whereas neutralization of HMGB1 significantly reduced these proinflammatory mediators. These results indicate that HMGB1 is crucial for triggering lung inflammation, whereas inhibition of HMGB1 promotes TGF-β yet inhibits proinflammatory cytokine programs during ALI.

Blocking HMGB1 Inhibits PTEN but Promotes β-Catenin Activation and Tregs in Acute Lung Injury

PTEN has been shown to promote inflammatory response by regulating macrophage activation (17). To test whether HMGB1 mediates PTEN activation in macrophages during lung injury, we collected alveolar macrophages from BALF after LPS instillation. Indeed, IgG control treatment significantly increased the expression of HMGB1 and PTEN in LPS-stimulated macrophages. However, blocking HMGB1 in LPS-stimulated macrophages significantly reduced PTEN and increased β-catenin expression (**Figure S2**, **Figures 2A,B**). Furthermore, HMGB1 neutralization decreased serum HMGB1 levels, as compared with controls (**Figure 2C**, 33.1 ± 13.1 vs. 71.2 ± 12.5, $p < 0.01$). Unlike in controls, TGF-β levels were elevated in anti-HMGB1 group (**Figure 2D**, 62.5 ± 11.3 vs. 25.3 ± 12.7, $p < 0.01$), accompanied by increased production of $CD4^+CD25^+Foxp3^+$ Tregs compared to IgG controls (**Figure S3**, **Figures 2E,F**, 6.86 ± 0.46 vs. 5.67 ± 0.88, $p < 0.05$) in the peripheral blood. These results indicate LPS-induced HMGB1 activates macrophage PTEN. Upon LPS treatment, HMGB1 blockade inhibits PTEN yet promotes β-catenin activation and induction of $CD4^+CD25^+Foxp3^+$ Tregs, which might be essential for the regulation of inflammatory response in LPS-induced ALI.

Myeloid Cell-Specific PTEN Is Critical for the HMGB1-Mediated Inflammatory Response in Acute Lung Injury

To determine whether myeloid cell-derived PTEN plays a role in HMGB1-mediated inflammatory response during lung injury, we used myeloid cell-specific PTEN knockout ($PTEN^{M-KO}$) mice as described (22). Indeed, increased animal survival was observed in $PTEN^{M-KO}$ mice, but not in $PTEN^{flox}$ control mice (**Figure 3A**, 83.5 vs. 46.6%, $p < 0.01$) at day 6 after LPS treatment. $PTEN^{M-KO}$ mice exhibited weight gain (**Figure 3B**, −11.9 to 1.2%, $p < 0.05$) compared to controls (−22.3 to −13.5%) from days 4–10. Unlike in $PTEN^{flox}$ controls, LPS-induced lung inflammation was attenuated in $PTEN^{M-KO}$ mice (**Figures 3C,D**, 2.02 ± 0.32 vs. 3.51 ± 0.45, $p < 0.01$). Using MPO activity assay, we found decreased lung neutrophil accumulation in $PTEN^{M-KO}$ mice after LPS stimulation, as compared with $PTEN^{flox}$ controls (**Figure 3E**, 0.67 ± 0.22 vs. 1.06 ± 0.77, $p < 0.05$). Similarly, $PTEN^{M-KO}$ increased animal survival (**Figure 3F**, 82.5% vs. 42.5% at day 6, $p < 0.01$) and body weight (**Figure 3G**, −6.5 to 1.2% vs. −18.3 to −8.8% from days 4–10, $p < 0.01$) in contrast to rHMGB1-treated $PTEN^{flox}$ controls. Treatment of $PTEN^{M-KO}$ mice with rHMGB1 reduced lung damage (**Figures 3H,I**, 2.59 ± 0.44 vs. 4.22 ± 1.27, $p < 0.01$), lung neutrophil accumulation (**Figure 3J**, 0.86 ± 0.45 vs. 2.55 ± 0.58, $p < 0.005$), and increased the expression of Foxp3 and TGF-β yet depressed RORγt, IL-17A, TNF-α, and IL-1β in lung tissues (**Figures 3K,L**,

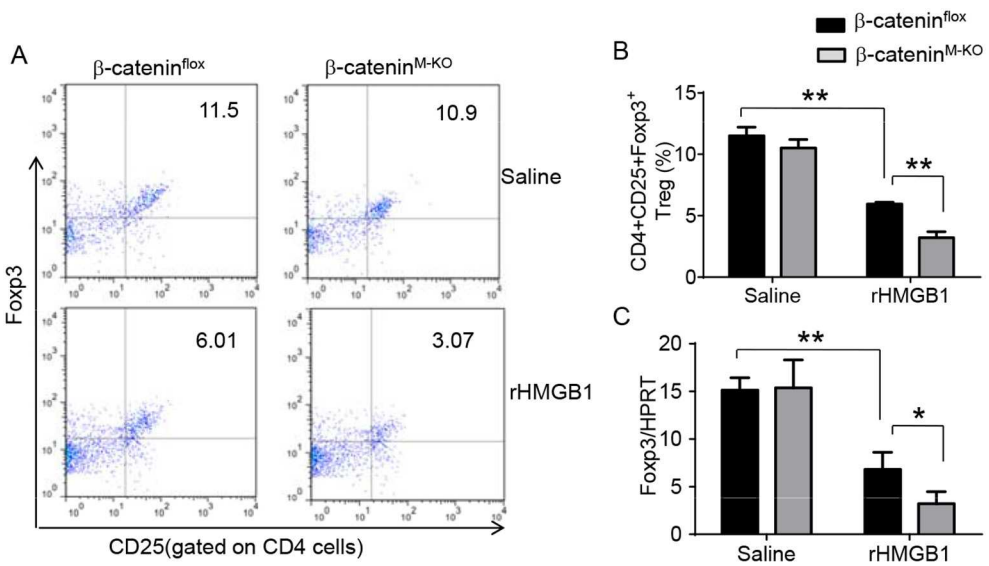

FIGURE 6 | Myeloid β-catenin deficiency reduces the frequency of $CD4^+CD25^+Foxp3^+$ Tregs and Foxp3 expression in lung injury. The $\beta\text{-catenin}^{flox}$ and $\beta\text{-catenin}^{M-KO}$ mice were given rHMGB1 at 24 h prior to blood harvest. **(A)** Representative diagrams of $CD4^+CD25^+Foxp3^+$ Tregs in the peripheral blood from $\beta\text{-catenin}^{flox}$ and $\beta\text{-catenin}^{M-KO}$ mice after rHMGB1 treatment was analyzed by flow cytometry. **(B)** The percentage of $CD4^+CD25^+Foxp3^+$ Tregs from $\beta\text{-catenin}^{flox}$ and $\beta\text{-catenin}^{M-KO}$ mice after rHMGB1 treatment ($n = 4$–6 animals/group), Mean ± SD, $^*p < 0.05$. **(C)** q-PCR analysis of mRNA expression coding for Foxp3 in peripheral blood T cells from $\beta\text{-catenin}^{flox}$ and $\beta\text{-catenin}^{M-KO}$ mice after rHMGB1 treatment. Mean ± SD ($n = 4$–6 samples/group), $^{**}p < 0.01$.

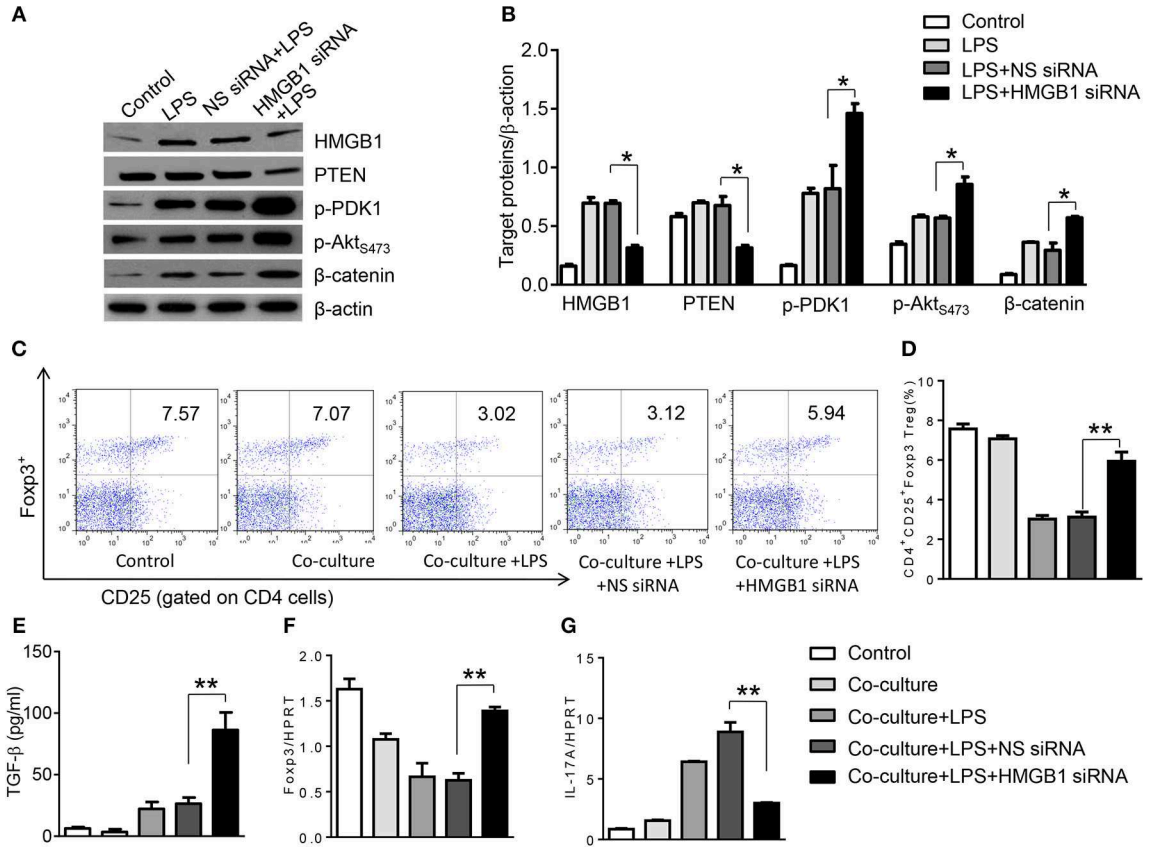

FIGURE 7 | HMGB1 blockade induces PDK1/Akt/β-catenin activation and CD4$^+$CD25$^+$Foxp3$^+$ Tregs *in vitro*. The peritoneal macrophages from WT mice was transfected with HMGB1 siRNA or NS siRNA, and then co-cultured with spleen T cells after LPS stimulation for 6 h. **(A)** The protein was isolated from HMGB1 siRNA- or NS siRNA-transfected macrophages in the co-cultures. The expression of PTEN, p-PDK1, p-Akt (ser473), and β-catenin was analyzed by Western blots. Representative of three experiments. **(B)** The density ratio of HMGB1, PTEN, p-PDK1, p-Akt (ser473), and β-catenin. $^*p < 0.05$, $^{**}p < 0.01$. **(C)** Representative diagrams of CD4$^+$CD25$^+$Foxp3$^+$Tregs in spleen T cells from co-cultures was analyzed by flow cytometry. **(D)** The percentage of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in spleen T cells from co-cultures (n = 4–6 samples/group), Mean ± SD, $^{**}p < 0.01$. **(E)** ELISA analysis of TGF-β levels in the supernatants from co-cultures. Mean ± SD (n = 4–6 samples/group). q-PCR analysis of mRNA expression coding for **(F)** Foxp3 and **(G)** IL-17A in spleen T cells from co-cultures. Mean ± SD (n = 4–6 samples/group), $^*p < 0.05$, $^{**}p < 0.01$.

$p < 0.01$). These findings suggest that myeloid PTEN is a critical mediator for HMGB1-induced inflammatory response during ALI.

Myeloid Cell-Specific PTEN Deficiency Activates β-Catenin Signaling and Treg Induction in Acute Lung Injury

We next test whether macrophage PTEN deficiency may affect β-catenin signaling and CD4$^+$CD25$^+$Foxp3$^+$ Treg induction *in vivo*. We found that myeloid PTEN deficiency increased phosphorylation of PDK1 and Akt, as well as β-catenin expression in peritoneal macrophages after rHMGB1 treatment, as compared with PTEN^{flox} controls (**Figures 4A,B**). The serum TGF-β levels were also increased in rHMGB1-treated PTEN^{M-KO} mice compared to controls (**Figure 4C**, 128.7 ± 37.7 vs. 35.1 ± 15.6, $p < 0.01$). In contrast to PTEN^{flox} T cells, we observed significantly increased frequency of CD4$^+$CD25$^+$Foxp3$^+$ Tregs (**Figures 4D,E**, 8.55 ± 0.77 vs. 4.61 ± 0.71, $p < 0.05$) in the peripheral blood, with substantially increased Foxp3 expression from rHMGB1-treated PTEN^{M-KO} mice (**Figure 4F**, $p < 0.01$). These findings implicate that macrophage PTEN deficiency can promote β-catenin signaling and CD4$^+$CD25$^+$Foxp3$^+$ Treg induction during lung inflammatory response.

Myeloid β-Catenin Signaling Is Essential for the Induction of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in Acute Lung Injury

To determine the role of β-catenin activation in producing CD4$^+$CD25$^+$Foxp3$^+$ Tregs, we used myeloid cell-specific β-catenin knockout (β-catenin^{M-KO}) mice. Indeed, animal survival rate was decreased in β-catenin^{M-KO} mice, but not in β-catenin^{flox} control mice (**Figure 5A**, 28.5 vs. 58.2%, $p < 0.01$) at day 6 after rHMGB1 treatment. The body weight was decreased in β-catenin^{M-KO} mice (**Figure 5B**, −25.5 to 3.2%, $p < 0.05$) compared to controls (−12.4 to 3.5%) from days 4–10. Unlike in β-catenin^{flox} controls, rHMGB1 treatment exacerbated lung injury in β-catenin^{M-KO} mice

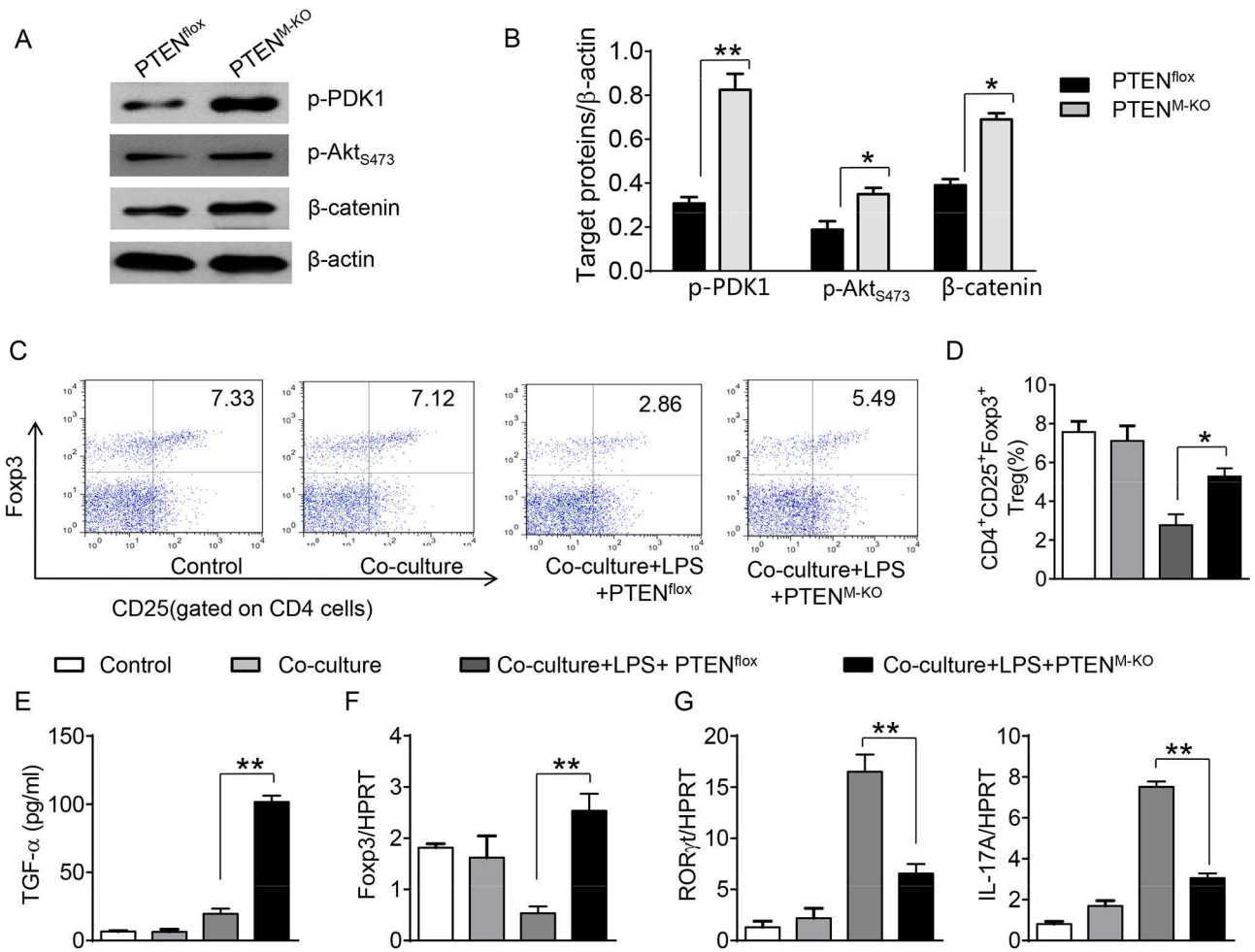

FIGURE 8 | Myeloid PTEN deficiency promotes β-catenin signaling and induces $CD4^+CD25^+Foxp3^+$ Tregs *in vitro*. The peritoneal macrophages were isolated from $PTEN^{flox}$ and $PTEN^{M-KO}$ mice, and then co-cultured with spleen T cells after LPS stimulation for 6 h. **(A)** The protein was isolated from macrophages in the co-cultures. The expression of p-PDK1, p-Akt (ser473), and β-catenin was analyzed by Western blots. Representative of three experiments. **(B)** The density ratio of p-PDK1, and p-Akt (ser473), β-catenin. $^*p < 0.05$. **(C)** Representative diagrams of $CD4^+CD25^+Foxp3^+$Tregs in spleen T cells from co-cultures was analyzed by flow cytometry. **(D)** The percentage of $CD4^+CD25^+Foxp3^+$ Tregs in spleen T cells from co-cultures ($n = 4$–6 samples/group), Mean ± SD, $^{**}p < 0.01$. **(E)** ELISA analysis of TGF-β levels in the supernatants from co-cultures. Mean±SD ($n = 4$–6 samples/group), $^*p < 0.05$. q-PCR analysis of mRNA expression coding for **(F)** Foxp3, **(G)** RORγt and IL-17A in spleen T cells from co-cultures. Mean ± SD ($n = 4$–6 samples/group), $^*p < 0.05$, $^{**}p < 0.01$.

(**Figures 5C,D**, 3.22 ± 0.98 vs. 5.59 ± 1.84, $p < 0.05$). MPO activity assay displayed an increased lung neutrophil accumulation in $\beta\text{-catenin}^{M-KO}$ mice after rHMGB1 treatment, as compared with $\beta\text{-catenin}^{flox}$ controls (**Figure 5E**, 4.97 ± 1.34 vs. 2.7 ± 0.58, $p < 0.05$). rHMGB1 treatment in $\beta\text{-catenin}^{M-KO}$ mice decreased TGF-β release (**Figure 5F**, $p < 0.001$) and Foxp3 expression (**Figure 5G**, $p < 0.05$) and yet augmented RORγt, IL-17A, TNF-α, and IL-1β (**Figure 5H**, $p < 0.05$) in lung tissues. Moreover, a reduced frequency of $CD4^+CD25^+Foxp3^+$ Tregs (**Figures 6A,B**, $p < 0.01$), accompanied by decreased Foxp3 expression in the peripheral blood (**Figure 6C**, $p < 0.05$) was observed in $\beta\text{-catenin}^{M-KO}$ mice after rHMGB1 treatment. These findings implicate that macrophage β-catenin deficiency reduces TGF-β release, Foxp3 expression, and $CD4^+CD25^+Foxp3^+$ Treg induction while increasing RORγt/IL-17A, implying the essential role of β-catenin in the mechanism of $CD4^+CD25^+Foxp3^+$ Treg induction during lung inflammatory response.

Disruption of the HMGB1-PTEN Axis Promotes β-Catenin Signaling and Induces Tregs *in vitro*

To further elucidate the potential mechanisms of the macrophage HMGB1/PTEN/β-catenin signaling in mediating Tregs during lung injury, we used the macrophage/spleen T cell co-culture system. We blocked HMGB1 with siRNA transfection in LPS-stimulated macrophages, and then co-cultured with spleen T cells. Indeed, HMGB1 knockdown decreased macrophage PTEN yet augmented p-PDK1, p-Akt, and β-catenin as compared

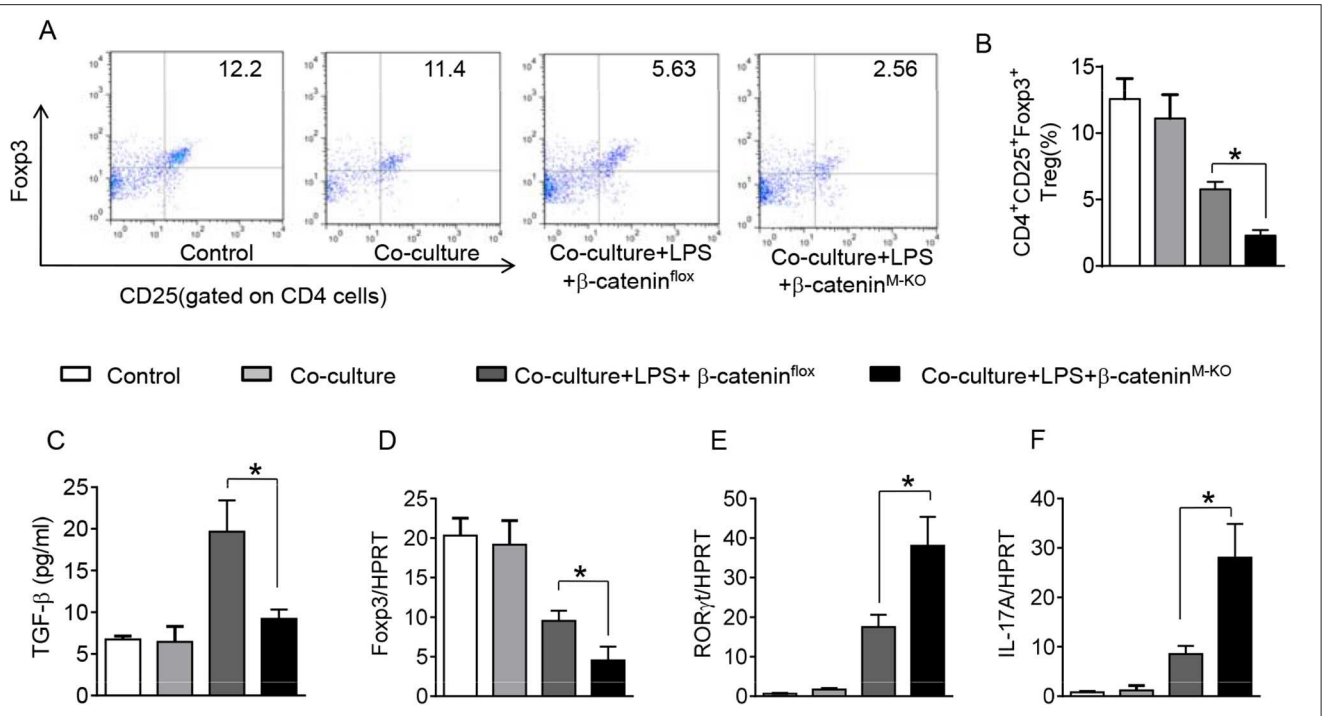

FIGURE 9 | Myeloid β-catenin signaling is essential for the induction of $CD4^+CD25^+Foxp3^+$ Tregs *in vitro*. The peritoneal macrophages were isolated from $\beta\text{-catenin}^{flox}$ and $\beta\text{-catenin}^{M-KO}$ mice, and then co-cultured with spleen T cells after LPS stimulation for 6 h. **(A)** Representative diagrams of $CD4^+CD25^+Foxp3^+$Tregs in spleen T cells from co-cultures was analyzed by flow cytometry. **(B)** The percentage of $CD4^+CD25^+Foxp3^+$ Tregs in spleen T cells from co-cultures ($n = 4$–6 samples/group), Mean ± SD. **(C)** ELISA analysis of TGF-β levels in the supernatants from co-cultures. Mean ± SD ($n = 4$–6 samples/group), $^*p < 0.05$. q-PCR analysis of mRNA expression coding for **(D)** Foxp3, **(E)** RORγt, and IL-17A **(F)** in spleen T cells from co-cultures. Mean ± SD ($n =$ 4–6 samples/group), $^*p < 0.05$.

with the NS siRNA-treated controls (**Figures 7A,B**). Staining spleen T cells from co-cultures by flow cytometry revealed significantly increased percentage of $CD4^+CD25^+Foxp3^+$ Tregs in HMGB1 siRNA-transfected cultures, compared to siRNA-treated controls (**Figures 7C,D**, 5.94 ± 0.55 vs. 3.12 ± 0.38, $p < 0.01$). Moreover, the HMGB1 knockdown significantly increased TGF-β levels in co-culture supernatants, compared to NS siRNA-treated controls (**Figure 7E**, 88.5 ± 26.3 vs. 26.8 ± 11.8, $p < 0.01$). Unlike control cultures, the Foxp3 expression was significantly increased, whereas IL-17A expression was suppressed in spleen T cells from HMGB1 siRNA-transfected co-cultures (**Figures 7F,G**). Furthermore, in contrast to $PTEN^{flox}$ controls, macrophage PTEN deficiency increased the expression of p-PDK1, p-Akt, and β-catenin (**Figures 8A,B**), accompanied by markedly increased percentage of $CD4^+CD25^+Foxp3^+$ Tregs (**Figures 8C,D**, 5.3 ± 0.43 vs. 2.7 ± 0.33, $p < 0.05$), TGF-β levels (**Figure 8E**, 94.6 ± 12.6 vs. 18.3 ± 4.5, $p <$ 0.01), and Foxp3 (**Figure 8F**) yet reduced RORγt and IL-17A expression (**Figure 8G**) in PTEN-deficient co-cultures. To confirm the importance of β-catenin signaling in the production of $CD4^+CD25^+$Foxp3 Tregs, we further analyzed the frequency of $CD4^+CD25^+Foxp3^+$ Tregs in spleen T cells after co-culturing with β-catenin-deficient macrophages. Indeed, a decreased percentage of $CD4^+CD25^+Foxp3^+$ Tregs (**Figures 9A,B**, $p <$ 0.05), with substantially reduced TGF-β levels (**Figure 9C**, $p <$ 0.05) and Foxp3 expression (**Figure 9D**, $p < 0.05$), yet increased RORγt and IL-17A expression (**Figures 9E,F**, $p < 0.05$) was observed in $\beta\text{-catenin}^{M-KO}$ co-cultures, as compared with $\beta\text{-catenin}^{flox}$ controls. Taken together, these findings indicate a potential mechanism by which disruption of HMGB1/PTEN axis activates β-catenin signaling and promotes TGF-β, which contributes to the induction of $CD4^+CD25^+Foxp3^+$ Tregs during lung injury in sepsis.

DISCUSSION

In this study, we have demonstrated, for the first time, that the HMGB1/PTEN/β-catenin signaling represents a novel regulatory pathway to induce $CD4^+CD25^+Foxp3^+$ Tregs in sepsis-induced lung injury.

Using the animal model of ALI, we found instillation of LPS triggered systemic inflammatory response and induced ALI, which was accompanied by induction of HMGB1. Though the exacerbated lung damage was shown in LPS instilled lungs, neutralization of HMGB1 with anti-HMGB1 antibody provided significant protection against ALI as evidenced by increasing animal survival and decreasing pulmonary edema. These findings are consistent with previous reports that intratracheal instillation of live bacterial or HMGB1 mediates an acute inflammatory response characterized by the development of pulmonary edema

and increased intrapulmonary production of proinflammatory cytokines (8, 17, 29).

Numerous studies have revealed the ability of $CD4^+CD25^+Foxp3^+$ Tregs to control immune responses in lung injury (13, 30–32). In a mouse model of LPS-induced ALI, we found that increased HMGB1 levels mitigated the accumulation of $CD4^+CD25^+Foxp3^+$ Tregs leading to exacerbated lung damage. Interestingly, increasing HMGB1 release and protein expression enhanced PTEN activation on alveolar macrophages after LPS instillation. However, neutralization of HMGB1 suppressed PTEN, which was accompanied by increased $CD4^+CD25^+Foxp3^+$ Tregs and reduced IL-17A in LPS-treated mice. Consistent with previous reports that deletion of PTEN enhanced the expansion of $CD4^+CD25^+$Tregs (33), our results indicate that PTEN might serve as a negative regulator of Treg peripheral homeostasis during lung inflammation.

Further evidence of PTEN-mediated modulation of Tregs in ALI was obtained from myeloid cell-specific PTEN knockout ($PTEN^{M-KO}$) mice. We found that, in contrast to the $PTEN^{flox}$ mice, $PTEN^{M-KO}$ mice treated with LPS or rHMGB1 had reduced lung injury, neutrophil accumulation, proinflmmatory mediators, and increased animal survival. Moreover, myeloid PTEN deficiency increased β-catenin expression and phosphorylation of PDK1 and Akt on macrophages, accompanied by increased peripheral Tregs and Foxp3 expression yet decreased RORγt and IL-17A. Since increasing release of HMGB1 induced macrophage PTEN activation, while deleting myeloid PTEN promoted Tregs, we believe that PTEN is a mediator in the modulation of innate and adaptive immunity during lung inflammation. Indeed, alveolar macrophages are essential for the initiation of innate immune response by binding the toll-like receptors (TLRs) (34). In response to TLRs, PTEN activation on macrophages triggers inflammatory response via regulating PI3K signaling (35, 36). Notably, our current data demonstrated that myeloid PTEN deficiency promoted β-catenin activation, consistent with our previous report that PTEN-mediated β-catenin signaling regulated Foxo1-TLR4 activation in lung inflammation (37), suggesting the endogenous innate immune signaling most likely contributes to the Treg induction. Indeed, expression of stabilized β-catenin controls Treg development and survival (38). Activation of β-catenin regulates inflammatory response and promotes anti-inflammatory mediator (39). Thus, our findings implicate that disruption of macrophage HMGB1 or PTEN, and activation of β-catenin may be a key pathway in the regulation of Treg development during lung injury.

The mechanisms underlying the macrophage HMGB1/PTEN/β-catenin signaling-mediated Treg induction appear to be complex during ALI. Our data showed that HMGB1 blockade or PTEN loss increased TGF-β release. However, reduced TGF-β release was observed from β-catenin deficient-macrophages in response to rHMGB1 stimulation. This is consistent with previous report that β-catenin was required for the TGF-β production to regulate immunity during inflammatory response (39). Indeed, TGF-β is a potent regulator of the immune and inflammatory system. *In vitro* stimulation of naïve $CD4^+$ T cells in the presence of TGF-β increased the expression of $CD4^+CD25^+Foxp3^+$ associated with *in vivo* suppressive activity during lung inflammatory response (40). Disruption of TGF-β impaired the development of $Foxp3^+$ Tregs and may lead to the multifocal inflammatory cell infiltration and multiorgan failure in mice (28, 41). Moreover, TGF-β inhibited RORγt activity and Th17 cell differentiation in human $CD4^+$ T cells (42). TGF-β-induced Foxp3 inhibited Th17 cell differentiation by regulating RORγt function (43). TGF-β promoted the development of Treg and expansion $Foxp3^+$-expressing $CD4^+CD25^+$ Tregs *in vivo* (44, 45). Lung-resident tissue macrophages can generate $Foxp3^+$ Tregs through increasing TGF-β expression (46). Consistent with this notion, we found increased TGF-β expression and secretion by alveolar macrophages were accompanied by increased $CD4^+CD25^+Foxp3^+$ Tregs and reduced RORγt/IL-17A after anti-HMGB1 treatment or myeloid PTEN deletion in our animal models. This implies that TGF-β may be essential for the induction of $CD4^+CD25^+Foxp3^+$ Tregs during HMGB1-induced inflammatory response. On the other hand, we found HMGB1 knockdown markedly inhibited macrophage PTEN expression in the co-culture system. This is consistent with deletion of myeloid PTEN, which increased the expression of PDK1, Akt, and β-catenin. Although PTEN deficiency increased the frequency of $CD4^+CD25^+Foxp3^+$ Tregs, ablation of myeloid β-catenin resulted in reduced $CD4^+CD25^+Foxp3^+$ Tregs and increased RORγt/IL-17A. Indeed, our previous study has shown that disruption of PTEN increased β-catenin, which in turn promoted PI3K/Akt signaling to native feedback to regulate TLR4-driven inflammatory response (47). Increased β-catenin activity enhanced TGF-β production on macrophages, whereas β-catenin deficiency lost the ability to produce TGF-β, myeloid cell motility and adhesion leading to impairing tissue repair (48). Hence, the HMGB1/PTEN/β-catenin signaling regulates Treg induction through multiple signaling pathways. Recent works indicated that PDK1, a downstream of PI3K signaling, plays an important role in the regulation of Treg function (21). PDK1 deficiency suppressed Treg accumulation while increasing IL-17-expressing population leading to enhancing inflammatory response (21). Activation of Akt by PDK1 phosphorylation promoted Tregs and enhanced their suppressive capacity to the Th17 cell differentiation (20). Furthermore, increased Akt phosphorylation enhanced β-catenin transcriptional activity (49). Activation of β-catenin is essential for the stimulation of Treg induction while inhibition of inflammatory T cells (39). These data are consistent with our results that activation of PDK1/Akt/β-catenin enhanced Treg induction and suppressed IL-17A transcription regulated by RORγt *in vitro* and *in vivo*. Although our current study was based on the primary ALI and it might have some modified signaling pathways with secondary ALI (systemic inflammation), our findings suggest that HMGB1/PTEN/β-catenin signaling is critical to contribute to the induction of $CD4^+CD25^+Foxp3^+$ Tregs in sepsis-induced lung injury.

In the present study, we observed that HMGB1 can be induced in endotoxin-stimulated macrophages during sepsis. HMGB1 induction activates PTEN and inhibits PI3K/PDK1/Akt leading to suppressed β-catenin activity, which then decreases TGF-β

release from macrophages, results in diminished $Foxp3^+$ Treg induction. Blockade of HMGB1 or macrophage PTEN deletion activates PI3K/PDK1/Akt and β-catenin signaling, which in turn enhances macrophage TGF-β leading to increased Foxp3 Treg induction while inhibiting Th17 cell differentiation during sepsis-induced lung injury.

In conclusion, the macrophage HMGB1/PTEN/β-catenin signaling displays a distinct capacity to regulate the development of $CD4^+CD25^+Foxp3^+$ Tregs during lung inflammation. Induction of Tregs ultimately alleviated inflammatory response and facilitated resolution of lung injury. By identifying the regulatory pathway of HMGB1/PTEN/β-catenin signaling on Treg induction, our studies provide the rationale for novel therapeutic strategies for treating sepsis-induced lung injury.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript is available, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The study protocol were approved by the Institutional Animal Care and Use Committee of Anhui Medical University (No: LLSC2013007).

AUTHOR CONTRIBUTIONS

MZ contributed to the experimental design, performed research, analyzed data, and wrote the first draft of manuscript. MD, RT, HL, ZG, and ZJ collected and analyzed the human samples. HF wrote and revised the manuscript. CL performed *in vitro* experiments. X-LC and BK contributed to the study concept, research design, data analysis, and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01612/full#supplementary-material

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Tbet Expression in Regulatory T Cells Is Required to Initiate Th1-Mediated Colitis

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In normal conditions gut homeostasis is maintained by the suppressive activity of regulatory T cells (Tregs), characterized by the expression of the transcription factor FoxP3. In human inflammatory bowel disease, which is believed to be the consequence of the loss of tolerance toward antigens normally contained in the gut lumen, Tregs have been found to be increased and functionally active, thus pointing against their possible role in the pathogenesis of this immune-mediated disease. Though, in inflammatory conditions, Tregs have been shown to upregulate the T helper (Th) type 1-related transcription factor Tbet and to express the pro-inflammatory cytokine IFNγ, thus suggesting that at a certain point of the inflammatory process, Tregs might contribute to inflammation rather than suppress it. Starting from the observation that Tregs isolated from the lamina propria of active but not inactive IBD patients or uninflamed controls express Tbet and IFNγ, we investigated the functional role of Th1-like Tregs in the dextran sulfate model of colitis. As observed in human IBD, Th1-like Tregs were upregulated in the inflamed lamina propria of treated mice and the expression of Tbet and IFNγ in Tregs preceded the accumulation of conventional Th1 cells. By using a Treg-specific Tbet conditional knockout, we demonstrated that Tbet expression in Tregs is required for the development of colitis. Indeed, Tbet knockout mice developed milder colitis and showed an impaired Th1 immune response. In these mice not only the Tbet deficient Tregs but also the Tbet proficient conventional T cells showed reduced IFNγ expression. However, Tbet deficiency did not affect the Tregs suppressive capacity *in vitro* and *in vivo* in the adoptive transfer model of colitis. In conclusion here we show that Tbet expression by Tregs sustains the early phase of the Th1-mediated inflammatory response in the gut.

Keywords: Treg cells, Tbet, Th1-like Tregs, inflammatory bowel disease, inflammation

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INTRODUCTION

In homeostatic conditions, the gastrointestinal tract is patrolled by immune cells which are in charge to fight against pathogens. At the same time, the intestinal mucosal immune system tolerates harmless antigens contained in the gut lumen. The unbalance between these pro-inflammatory and tolerogenic activities is believed to cause inflammatory bowel disease (IBD), whose main forms are

Crohn's disease (CD) and ulcerative colitis (UC). In IBD, antigens normally contained in the gut lumen derived from the gut microbiota or introduced with diet, cause an excessive activation of the immune system leading to chronic inflammation and irreversible tissue damage (1).

Regulatory T cells (Tregs) are a class of T cells which negatively control the pro-inflammatory activity of adaptive and innate immune cells and they play a key role in the maintenance of the gut immune homeostasis (2). In the absence of Tregs, as observed in case of loss-of-function mutations of the Tregs lineage committing transcription factor *foxp3* (i.e., IPEX syndrome in humans, *scurfy* mice) (3, 4), or in case of functional mutations of genes encoding for molecules involved in the Tregs suppressive activity such as CTLA4 and IL10, an uncontrolled activation of the immune system in the gut is invariantly observed (5–7).

Despite the pivotal role of Tregs in the maintenance of gut homeostasis, the number of Tregs is not reduced in the lamina propria of IBD patients and they result even increased in the inflamed areas (8, 9). Moreover, Tregs isolated from IBD patients were shown to be as suppressive as Tregs isolated from non-IBD controls (10). Therefore, the role of Tregs in the pathogenesis of IBD remains elusive.

Although considered finally differentiated cells, Tregs have been recently shown to have a certain functional plasticity. For instance, Tregs can acquire the expression of the master transcription factors that define the T helper cell subsets they are suppressing (11, 12). In this context, the expression of Tbet by Tregs, as observed in Th1 immune responses induced by *Mycobacterium* sp. infection, has been shown to induce the expression of the chemokine receptor CXCR3 and to promote the Tregs homing at the site where Th1 cells need to be kept in check (13).

Although Tbet expressing Tregs do not normally secrete pro-inflammatory cytokines and maintain their suppressive capacity, in certain conditions they can acquire a Th1-like phenotype characterized by IFNγ secretion and pro-inflammatory functions (14, 15). Moreover, several reports indicate that IFNγ -expressing Th1-like Tregs are involved in the pathogenesis of different inflammatory diseases (16–18). Although IFNγ expressing Th1-like Tregs have been described in models of intestinal inflammation (19, 20), the presence of these cells in human IBD and their functional role in intestinal inflammation remain unclear.

Here, we provide evidence that IFNγ-expressing cells accumulate in the inflamed tissue of both CD and UC patients. We also demonstrate that mice developing chemically-induced colitis are characterized by an increased number of IFNγ-expressing Th1-like Tregs in the intestinal lamina propria and that their upregulation precedes the accumulation of conventional Th1 cells. Finally, by generating Treg-specific Tbet conditional knockout mice, we demonstrate that pro-inflammatory Th1-like Tregs are required for the development of intestinal inflammation.

MATERIALS AND METHODS

Patients

Intestinal biopsies of IBD patients (Ileal CD $n = 8$; colonic CD $n = 5$; active UC $n = 7$; inactive UC $n = 5$), and patients undergoing intestinal surgical resection for pathologies unrelated to IBD, including intestinal tumors (ileal controls $n = 7$; colonic controls $n = 5$) were obtained from the Policlinico Tor Vergata, Rome, Italy and IRCCS Ospedale Maggiore Policlinico di Milano, Milan, Italy. The clinical characteristics and concomitant therapies of IBD patients are summarized in the **Supplementary Table 1**. Disease extent of UC and localization and behavior of CD were described according to the Montreal classification for IBD. Local Ethics Committees (Tor Vergata University Hospital, Rome. Protocol number:154/12).

Mice

All mice used were on C57BL6 genetic background and were housed and bred under specific pathogen-free conditions in a facility located in Castel Romano, Rome. To generate the FoxP3 reporter mice, FoxP3eGFP-Cre knock-in mice (kindly provided by A. Rudensky, MSKCC, NY, USA) which bear the coding sequence for the green fluorescent protein/iCre-recombinase fusion protein knocked-in the FoxP3 locus were crossed with the Rosa26-tdRFP mice (kindly provided by Hans Joerg Fehling, University of Ulm, Germany). tdRFP mice bear a mutation consisting in a reporter allele for Cre activity that expresses a non-toxic tandem-dimer red fluorescent protein (tdRFP) following Cre-mediated deletion of a floxed neo/stop cassette. Treg-specific Tbx21 conditional knockout mice were generated crossing FoxP3 reporter mice with mice bearing the floxed Tbx21 allele ($Tbx21^{fl/fl}$ JAX, Bar Harbor, ME, USA). In these mice, Cre expression, under control of the FoxP3 promoter, cause the deletion of Tbx21 floxed exons leading to gene inactivation selectively in Tregs. Both FoxP3 reporter and Treg specific Tbet conditional knockout mouse strains were vital and born in the expected mendelian ratios with sign of spontaneous disease up to 12 months. IFNγ knockout mice were purchased from Jackson Laboratories (JAX, Bar Harbor, ME, USA). All animal experiments were performed in accordance with the local institutional guidelines. Male mice (6–8 weeks old) were used for all the experiments.

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Experimental Colitis

Chemically-induced colitis was induced in FoxP3 reporter, conditional Tbx21 knockout and IFNγ knockout mice. Mice were treated with 2% dextran sulfate sodium (DSS, TdB Consultancy AB, Uppsala, Sweden) in drinking water for 7 days followed by normal drinking water until day 10. Body weight was monitored daily.

Adoptive transfer model of colitis was performed by injecting i.p. 6–8 weeks old RAG1-deficient mice with 4×10^5 FACS sorted $CD4+CD45RB^{high}$ cells from splenocytes of wild type C57BL6

mice alone or together with the same number of Tregs isolated from the spleen of FoxP3^{Cre}Tbx21$^{wt/wt}$ or FoxP3CreTbx21$^{fl/fl}$ mice. Body weight was monitored every other day.

Endoscopic Procedures

The development of DSS-induced colitis in mice was assessed by micro-endoscopy as previously described (21).

Histologic Analysis of Colon Cross Sections

Colonic cross sections were stained with H&E, and the severity of inflammation was evaluated as previously described (22).

Isolation of Leukocyte Subpopulations and Flow Cytometry Analysis

Human lamina propria mononuclear cells (LPMC) were isolated according to standard protocols. Briefly, the dissected intestinal mucosa was freed of mucus and epithelial cells in sequential steps with DTT (0.1 mmol/L) and EDTA (1 mmol/L) (both from Sigma, Milan, Italy) and then digested with collagenase D (400 U/ml) (Worthington Biochemical Corporation, Lakewood, NJ) for 5 h at 37°C. LPMC were then separated with a Percoll gradient and cultured in complete RPMI 1640 medium containing 5% human serum (Sigma, Milan, Italy) and 100 U/ml IL-2 (Proleukin, Novartis, Switzerland). Cells suspension was stained with anti-CD4 clone RPA-T4 (Biolegend, San Diego, California), anti-Foxp3 clone PCH101 (eBioscience, Thermo Fisher scientific, Italy), anti-Tbet clone 04-46 (BD Pharmigen, Milan, Italy) and anti IFNγ clone 4s.b3 (Biolegend, San Diego, California) after 5 h stimulation with 40 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin (Sigma Aldrich, Milan, Italy), in the presence of 2 nmol/l monensin (eBioscience, Thermo Fisher, Italy) according to standard protocols.

Mouse LPMC were obtained according to Lamina Propria Dissociation Kit mouse protocol (Miltenyi Biotech, Bergisch-Gladbach, Germany). Colonic Lamina Propria Mononuclear Cells (LPMC) were stained with LIVE/DEAD® staining (Life Technology, Milan, Italy) and with surface fluorochrome-conjugated antibodies against CD3 clone 500A2, CD4 clone GK1.5 (BD Pharmingen, Milan, Italy). Permeabilization and intracellular staining with conjugated anti-Tbet clone Q313778, anti-IFNγ clone XMG1.2 or anti-IL10 clone JES5-16E3 (BD Pharmingen, Milan, Italy), were performed after 5 h stimulation with PMA/ionomycicn. Cells were acquired with a FACS VERSE® (BD Bioscience, San Jose, CA) gating on living cells and the data were analyzed with FlowJo software (BD).

In vitro Induction of Th1-Like Tregs

In some experiments CD4+ T cells were magnetically sorted from splenocytes by using the CD4+ T Cell Isolation kit mouse (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacture's protocol. At least 97% purity was verified by flow cytometry before each experiment. To induce Tbet and IFNγ expression, CD4+ T cells were polyclonally activated in RPMI 1640 10% FBS (Lonza, Basel, Switzerland) with plate bound anti-CD3 (clone 145-2C11, eBioscience, Thermo Fisher scientific, Italy) and 1 mg/ml anti CD28 (clone 37.31, eBioscience, Thermo Fisher scientific, Italy). In some experiment CD4+ T cells were cultured in the presence of 10 mg/ml anti mouse IFNγ neutralizing antibody (clone XMG1.2, eBioscience, Thermo Fisher scientific, Italy) or the isotype control IgG2a. FACS sorted FoxP3+ Tregs were cultured in RPMI1640 10% FBS for 24 h in the presence of plate bound anti-CD3, 10 μg/ml anti-CD28, 20 ng/ml mouse recombinant (mr)IL2 (R&D Systems, Abingdon, UK) and 100 ng/ml mrIFNγ or 15 ng/ml mrIL12 or 10 ng/ml mrIL23 or 20 ng/ml mrIL6 or 50 ng/ml mrIL21 (R&D Systems, Abingdon, UK).

Cell Proliferation Assay

105 FACS-sorted CD4+CD45RBhigh responder (R) cells were labeled with CellTrace®-violet (Thermo Fisher scientific, Italy) according to manufacturer's instruction and co-cultured with sorted CD4+FoxP3+ Tregs suppressor (S) from splenocytes of FoxP3^{Cre}Tbx21$^{wt/wt}$ or FoxP3CreTbx21$^{fl/fl}$ at different ratios in the presence of plate bound anti-CD3 clone 145-2C11 (BD Pharmigen, Milan, Italy) and 10^4 sorted CD90- splenocytes used as antigen-presenting cells. CellTrace® fluorescence was evaluated after 5 days co-culture and the fraction of non-proliferating cells used as suppression index.

RNA Extraction, Complementary DNA Preparation, and Real-Time PCR

Total RNA was isolated with the PureLink® RNA Micro Kit (Thermo Fisher scientific, Italy) for *in vitro* experiments and with PureLink® RNA Mini Kit (Thermo Fisher scientific, Italy) for tissue, according to the manufacturer's recommendations. Reverse transcription into cDNA was performed with the Superscript III Reverse Transcriptase kit (Invitrogen, Thermo Fisher scientific, Italy) according to the manufacturer's protocol and then amplified by real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy). PCR was performed by using the primers described in the **Supplementary Table 2**. RNA expression was calculated relative to the housekeeping beta-actin gene expression on the base of the ΔCt algorithm.

Statistics

The Mann Whitney test and the unpaired student's *t*-test were used to evaluate differences between the two experimental groups after checking for normal distribution of data. Statistically significant differences between groups are indicated ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Statistical analysis was done performed using GraphPad Prism software (Graphpad Software Inc., San Diego, CA, USA).

RESULTS

Th1-Like Tregs Are Up-Regulated in Active IBD

In a first set of experiments we aimed to assess the presence of Th1-like Tregs, characterized by the expression of Tbet and IFN-γ in the gut of IBD patients. To this end, mucosa biopsies were collected from the most inflamed intestinal areas of UC ($n = 7$) and CD ($n = 8$) patients. Biopsies from inactive UC ($n = 5$) were

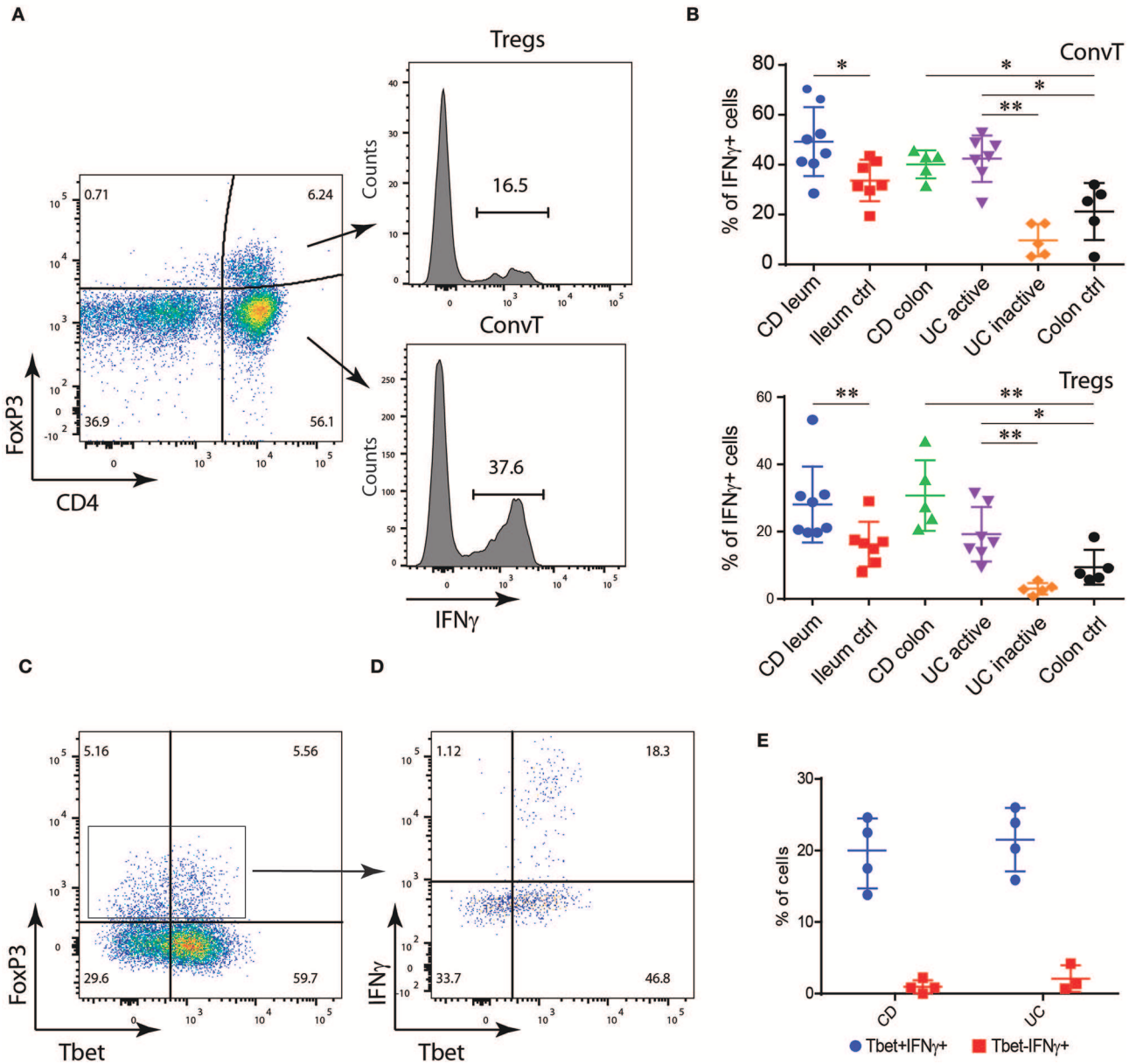

FIGURE 1 | Lamina propria (LP) FoxP3+CD4+ T cells from IBD patients express IFNγ. **(A)** Flow cytometric analysis of IFNγ expression in CD4+FoxP3+ regulatory T cells (Tregs) and CD4+FoxP3- conventional T cells (ConvT) cells from LP cells of IBD patients and controls: gating strategy. **(B)** Flow cytometric analysis of IFNγ expression in Tregs (upper panel) and ConvT (lower panel) from ileal Crohn's disease (CD; $n = 8$), control ileum ($n = 7$), colonic CD ($n = 5$), active ulcerative colitis (UC; $n = 7$), inactive UC ($n = 4$), and control colon ($n = 5$). **(C,D)** Representative dot plot representing FoxP3 and Tbet expression in LP CD4+ T cells **(C)** and Tbet and IFNγ expression in LP FoxP3+CD4+ T cells **(D)** from IBD patients. **(E)** Frequency of IFNγ+ cells among Tbet+ and Tbet- Tregs in CD ($n = 4$) and UC ($n = 3$) patients. Numbers in the dot plot quadrants and histograms represent the relative frequency of cell subpopulations. Horizontal bars in **(B,D)** represent the mean value ±SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

also collected. As controls, samples of normal ileal ($n = 7$) and colonic mucosa ($n = 5$) were taken from patients undergoing surgical resection for colonic neoplasia. As shown in **Figure 1A**, IFNγ was expressed in both FoxP3 negative conventional T cells (ConvT) and Foxp3+ Tregs. As expected, ConvT cells from CD patients expressed more IFNγ as compared to controls. Similarly, ConvT cells from active UC expressed more IFNγ as compared to both inactive UC and control samples (**Figure 1B**, upper panel). Concerning Tregs, about 30% of the FoxP3+ cells from the inflamed ileal and colonic mucosa of CD patients expressed IFNγ and their frequency was significantly higher than their relative controls. In UC, the frequency of Tregs-expressing IFNγ was

about 20% and this was significantly higher than that observed in both inactive UC patients and colonic controls (**Figure 1B**, lower panel). Similarly to ConvT cells, a proportion of Foxp3+Tregs from inflamed IBD co-expressed the Th1-related transcription factor Tbet (**Figure 1C**) but IFNγ was exclusively expressed by Tbet+ Tregs in both CD and UC (**Figures 1D,E**). These results suggest that Th1-like Tregs, characterized by the expression of Tbet and IFNγ, might play a role during the inflammatory flares in IBD patients.

Th1-Like Tregs Are Upregulated in the Early Phase of Inflammation in the DSS Model of Colitis

To investigate the functional role of Th1-like Tregs in the development of intestinal inflammation, we first assessed whether Tbet-expressing Tregs were generated during intestinal inflammation induced in mice after oral administration of dextran sodium sulfate (DSS). This model is characterized by the induction of a potent Th1 immune response secondary to the damage of the intestinal epithelial barrier and increased permeability to luminal antigens. To this end, we used FoxP3$^{GFP-Cre}$Rosa26^{tdRFP} fate mapping reporter mice. In these mice, Tregs are characterized by the double expression of eGFP and tdRFP fluorescence (**Supplementary Figures 1A,B**). Reporter mice were treated with DSS for 7 days and sacrificed at day 10, when the peak of inflammation occurred (**Supplementary Figure 1C**). At the end of the experiment, Tbet-expressing Tregs increased in the lamina propria of DSS treated mice as compared to untreated controls (**Figure 2A**). Interestingly, in this early phase of colitis, the frequency of Tbet positive cells among Treg but not eGFP/tdRFP double negative ConvT cells increased during inflammation (**Figure 2B**). However, in terms of absolute numbers, both Tbet+ ConvT and Treg cells resulted increased in treated mice as compared to controls (**Figure 2C**) thus suggesting that the accumulation of Tbet-expressing Tregs in the lamina propria during the initial phase of inflammation might be proportionally greater than that of ConvT cells. Accordingly, the number of Tbet+ Tregs represented one third of all the CD4+ Tbet+ cells infiltrating the lamina propria at this time point as compared to one tenth in the untreated mice. Since IFNγ expression distinguishes Tbet-expressing Tregs with Th1-specific suppressive capacity from Th1-like pro-inflammatory Tregs, we assessed IFNγ expression by lamina propria Tregs in our model. The frequency of IFNγ positive cells resulted increased among Tregs in DSS treated mice as compared to the untreated controls (**Figure 2D**). Similarly to Tbet expression, the frequency of IFNγ -positive cells remained stable among ConvT cells (**Figure 2E**) while the absolute number of both ConvT and Treg cells expressing IFNγ resulted increased in the treated mice, the Th1-like Tregs representing one third of the total IFNγ-secreting CD4+ T cells (**Figure 2F**). Taken together, these data indicate that in a colitis model characterized by the alteration of the epithelial barrier, Tregs acquire a Th1-like phenotype characterized by Tbet and IFNγ expression. In order to investigate how early Th1-like Tregs appeared during colitis development, we performed a time course experiment in which mice were sacrificed at day 3, 6, and 10 of the DSS protocol. At day 3 and 6, DSS treated mice were characterized by none or mild colitis as shown by histological score of colitis severity (**Figures 3A,B**). The frequency of Tbet-expressing Tregs but not ConvT cells increased by day 6 as compared to untreated mice when only mild signs of colitis were observed (**Figures 3C,D**) prompting us to hypothesize that Th1-like Tregs might represent a "ready-to-use" source of IFNγ supporting the early stage of Th1-mediated immune response.

IFN-γ Plays a Non-redundant Role in the Induction of Th1-Like Tregs Both *in vitro* and *in vivo*

To test the hypothesis that Tbet induction and IFNγ expression in Tregs might be induced during T cell activation to support the development of Th1 cells, total CD4+ T cells sorted from the spleen of FoxP3$^{GFP-Cre}$Rosa26^{tdRFP} were polyclonally activated *in vitro* for 48 h or left unstimulated, and Tbet and IFNγ expression evaluated by flow cytometry. Activated but not resting cells upregulated Tbet and IFNγ in both Treg and ConvT cells (**Figure 4A**). Tbet resulted upregulated as early as 12 h after stimulation in both Tregs and ConvT and their relative number progressively increased reaching 85 and 60%, respectively, at 48 h (**Figure 4B**). Tbet expression was higher among Tregs than ConvT cells at each time point. IFNγ was expressed by a small fraction of cells after 12 and 24 h and increased at 48 h (**Figure 4C**). At this time point, 65 and 25% of Tregs and ConvT cells, respectively, resulted IFNγ positive. These results indicate that in the gut, Tregs are poised to rapidly upregulate Tbet and IFNγ after activation. IFNγ has been shown to induce the expression of Tbet in FoxP3+ cells. Accordingly, sorted FoxP3+ Tregs failed to upregulate Tbet after activation in the absence of IFNγ, while the addition of IFNγ to the cell culture medium but not other cytokines (i.e., IL12, IL23, IL6, or IL23), induced Tbet expression (**Supplementary Figure 2A**). To assess whether IFNγ was required to induce Tbet expression in our *in vitro* system, CD4+ T cells were activated in the presence or absence of neutralizing anti-IFNγ antibodies. In the presence of anti-IFNγ, Tbet expression and IFNγ secretion were reduced in both Tregs and ConvT, being the suppressive effect prevalent in the latter (**Figure 5A**). Moreover, the magnitude of IFNγ suppression among ConvT cells resulted bigger than the one observed in Tregs at each of the analyzed time points (**Figures 5B,C**).

To confirm *in vivo* the role of IFNγ in the induction of Th1-like Tregs during colitis, IFNγ^{het} and IFNγ^{Ko} mice underwent the DSS protocol and LPMC were analyzed at day 10. As previously reported, IFNγ^{Ko} mice developed milder disease as shown by the lower weight loss as compared to the IFNγ^{Het} control mice [**Supplementary Figure 2B**; (23)]. At the end of the experiment, Tbet expression resulted suppressed in the lamina propria Tregs of IFNγ^{Ko} mice as compared to the heterozygous controls (**Figure 5D**) thus demonstrating that IFNγ plays a non-redundant role in the induction of Th1-like Tregs during intestinal inflammation.

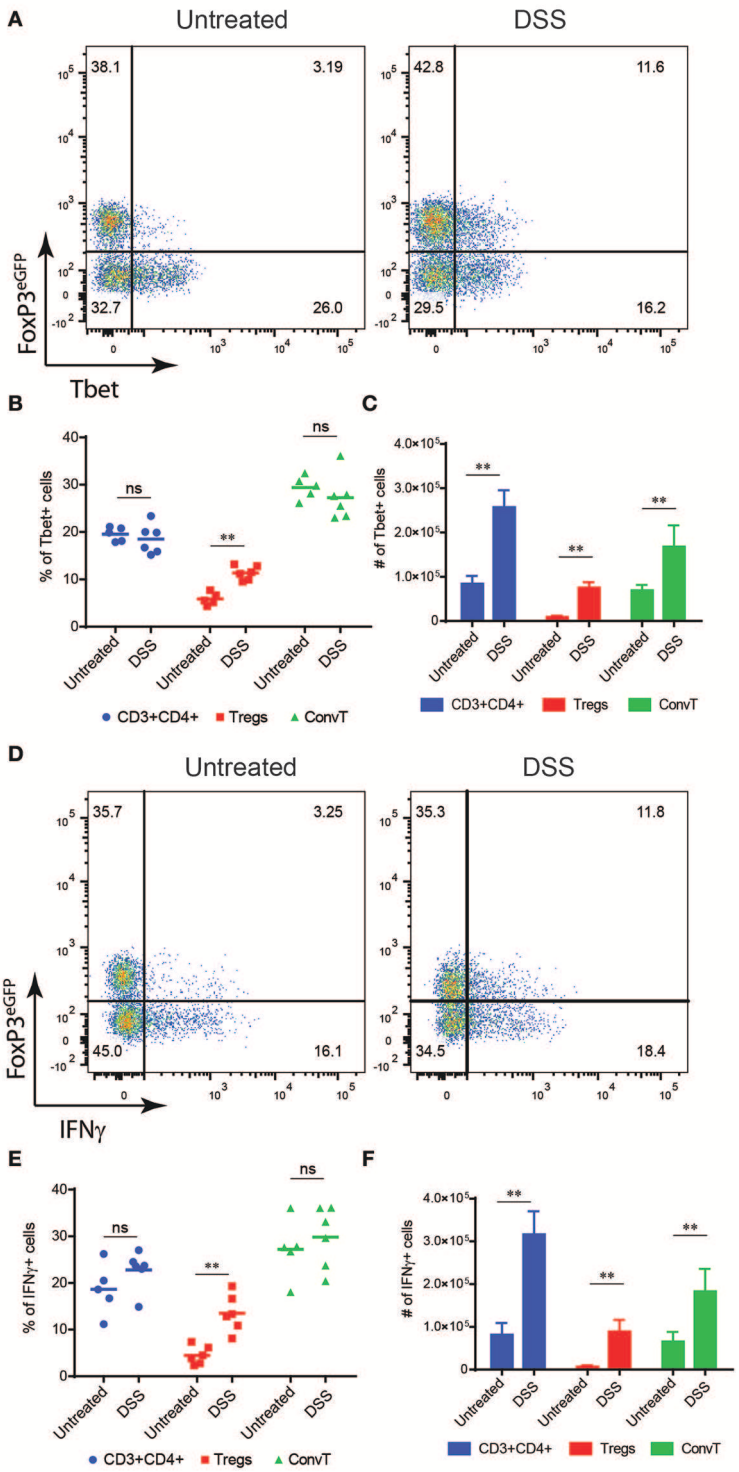

FIGURE 2 | (A) Representative dot plots showing FoxP3^{eGFP} and Tbet expression in LP CD3+CD4+ T cells isolated from untreated and DSS-treated mice. Frequency **(B)** and absolute numbers **(C)** of Tbet+ cells among total CD4+, Treg and ConvT cells as indicated. **(D)** Representative FoxP3^{eGFP} and IFNγ expression of the same cells as in **(A)**. Frequency **(E)** and absolute **(F)** numbers of IFNγ+ cells among total CD4+, Treg, and ConvT cells as indicated. Numbers in the dot plot quadrants represent the relative frequency of cell subpopulations. Horizontal bars indicate the mean, symbols indicate each analyzed mouse. Vertical bars indicate mean ±SEM. $^{**}p < 0.01$, ns, not significant.

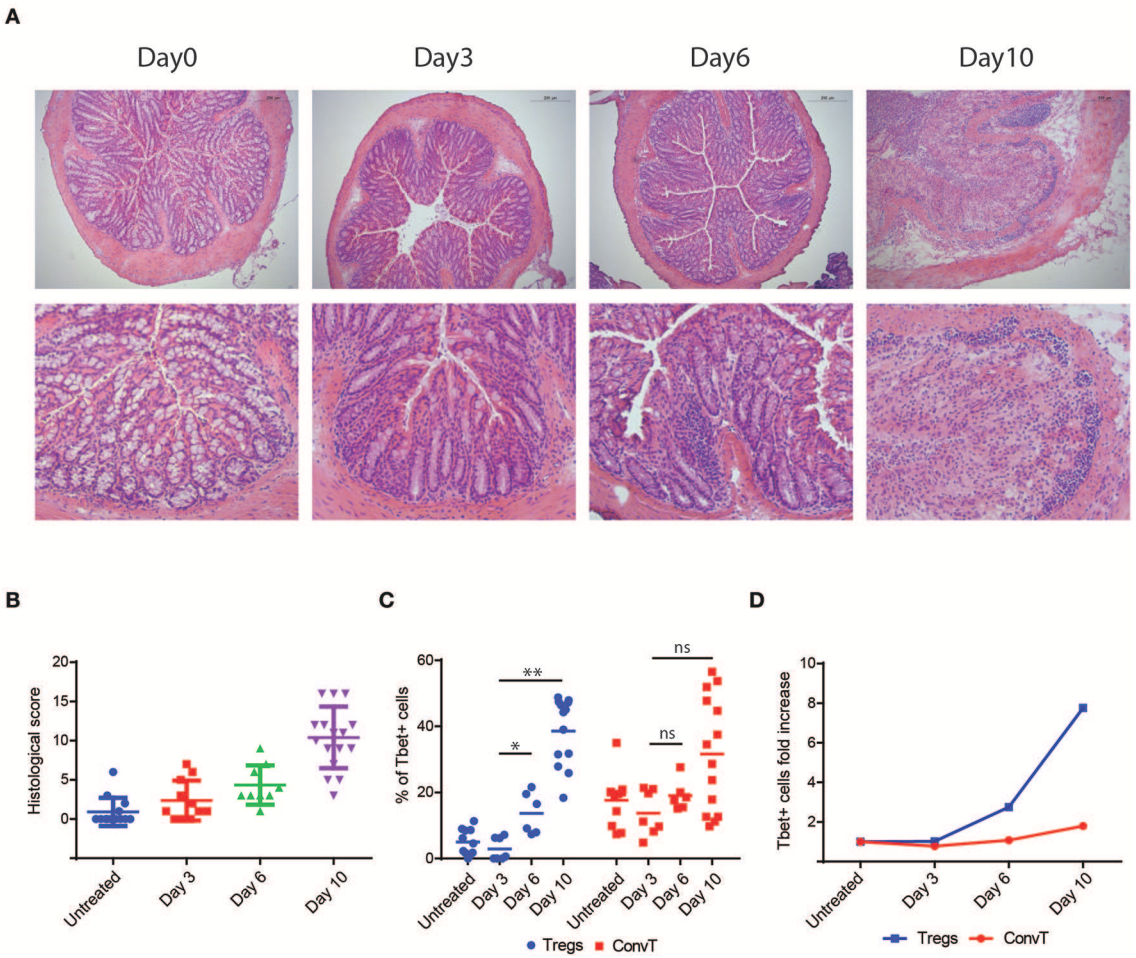

FIGURE 3 | (A) Representative histological sections of colons from DSS-treated mice (upper panel 20x magnification, lower panel 40x magnification) at different time points as indicated. Bars in the pictures indicate the scale. **(B)** Cumulative histologic score of DSS-treated mice pooled from three independent experiments at different time points, bars indicate the mean ±SD. **(C)** Percent of Tbet+ cells among LP Tregs and ConvT cells from DSS-treated mice at different time points, symbols indicate each analyzed mouse, bars indicate the mean. **(D)** Tbet+ cells fold increase relative to baseline (day 0) in Tregs and ConvT cells. Numbers in the dot plot quadrants represent the cell relative frequency. *$p < 0.05$; **$p < 0.01$; ns, not significant.

Th1-Like Tregs Are Required for the Development of DSS Colitis

To assess the role of Th1-like Tregs n the development of colitis, we crossed FoxP3$^{eGFP-Cre}$Rosa26^{tdRFP} mice with mice carrying floxed *TBX21* alleles (TBX21$^{fl/fl}$) to obtain a Treg-specific Tbet conditional knockout mouse (from here on indicated as FoxP3^{Cre}TBX21$^{fl/fl}$). FoxP3^{Cre}TBX21$^{fl/fl}$ and the FoxP3^{Cre}TBX21$^{wt/wt}$ mice, used as control, were treated with DSS as described above. FoxP3^{Cre}TBX21$^{fl/fl}$ mice showed milder weight loss and lower endoscopic grade of colitis severity as compared to FoxP3^{Cre}TBX21$^{wt/wt}$ mice (**Figures 6A,B**). Accordingly, at the end of the experiment, FoxP3^{Cre}TBX21$^{fl/fl}$ mice were characterized by milder colitis than controls, as shown by the histology score (**Figure 6C**) and the lower expression of the neutrophil-related marker *lcn-2* mRNA (**Figure 6D**). The cytokine profile analysis showed reduced *ifnγ* and *tnfα* mRNA expression while no significant differences were observed in *il17a*, *il6*, *il22,* and *il10* expression (**Figure 6E**). *ifnγ* and *tnfα*, were the most downregulated cytokines in FoxP3^{Cre}TBX21$^{fl/fl}$ mice, with a reduction by 10 and 5-fold respectively (**Figure 6F**). Finally, *il12p35* but not *il23p19* were reduced in knockout mice as compared to control mice (**Figure 6G**). These data indicate that the reduced inflammation observed in FoxP3^{Cre}Tbx21$^{fl/fl}$ mice was caused neither by a defect in the Th17-mediated immune response nor to an increased expression of anti-inflammatory cytokines but rather by a reduced expression of Th1-related cytokines. Since the frequency of Tregs, ex-Tregs and ConvT, based on the expression of the endogenous fluorescence eGFP and tdRFP, did not differ between Tbet knockout mice and the controls (**Supplementary Figure 3A**), to investigate whether the milder inflammatory phenotype shown by FoxP3^{Cre}Tbx21$^{fl/fl}$ was due to the increased suppressive capacity of Tregs in the absence of Tbet, Tregs from FoxP3^{Cre}Tbx21$^{fl/fl}$ and FoxP3^{Cre}Tbx21$^{wt/wt}$

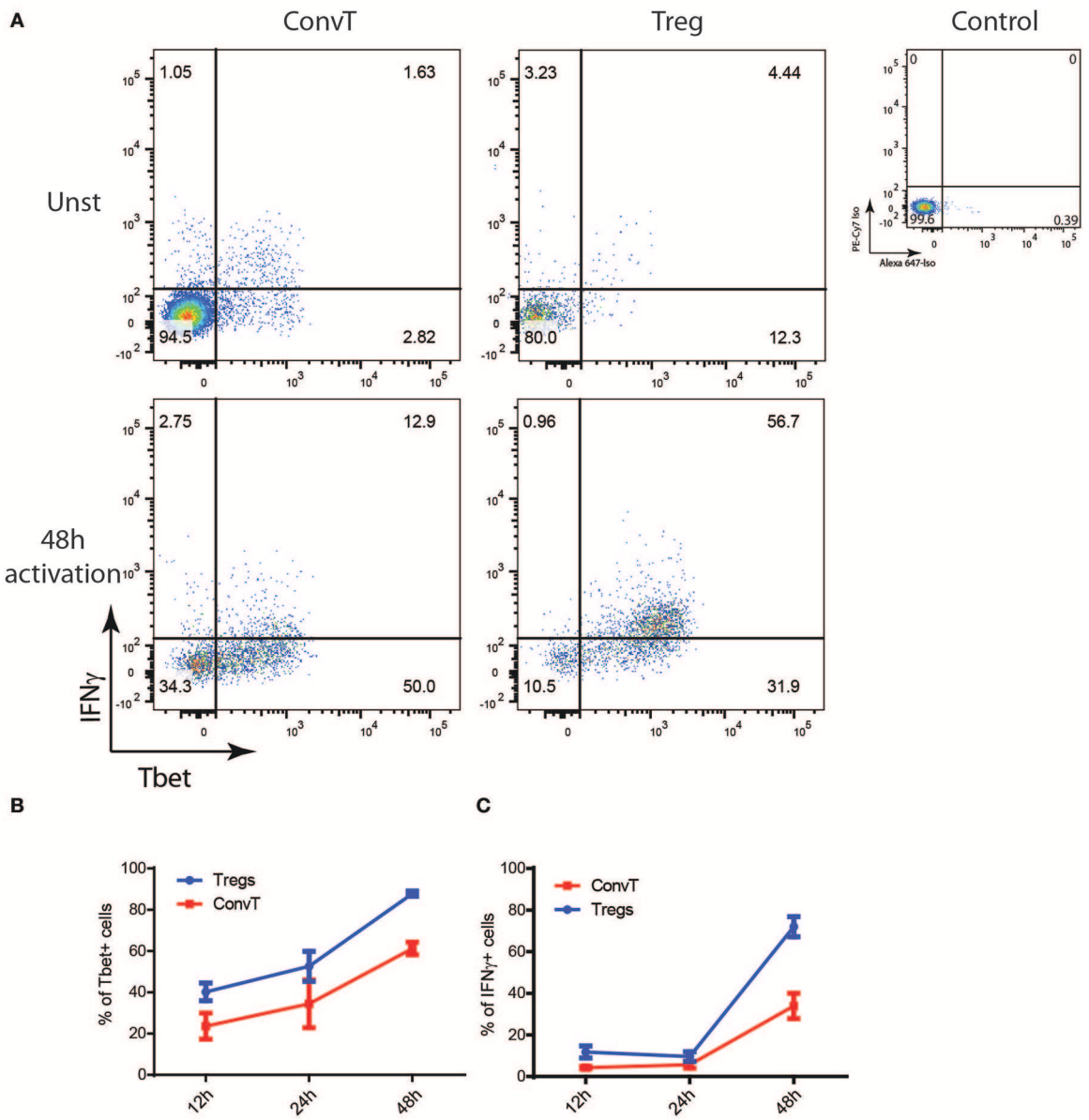

FIGURE 4 | Total CD3+CD4+ T cells from the spleen of reporter mice were polyclonally activated *in vitro*. **(A)** Representative dot plots representing Tbet and IFNγ expression gating on Tregs and ConvT after 48 h of stimulation. Tbet and IFNγ isotype control stainings gating on CD4+ cells are shown (upper right plot). Percent of Tbet+ **(B)** and IFNγ+ **(C)** cells among Treg and ConvT cells at different time points. Each point represents the mean ±SD of three independent experiment performed.

were co-cultured with CellTrace®-violet-labeled wild type CD45RB^{high} naïve T cells used as responder cells. In these experiments no difference in the suppressive capacity of wild type and Tbet knockout Tregs cells was observed at each responder-to-suppressor cell ratios (**Supplementary Figure 3B**). No difference was also observed when Tregs from FoxP3^{Cre}Tbx21$^{fl/fl}$ and FoxP3^{Cre}Tbx21$^{wt/wt}$ were used *in vivo* in the adoptive transfer model of colitis (**Supplementary Figure 3C**) as previously reported (24, 25). Finally, the frequency of IL10-expressing Tregs isolated from the colon of either DSS-treated FoxP3^{Cre}Tbx21$^{fl/fl}$ or FoxP3^{Cre}Tbx21$^{wt/wt}$ did not differ (**Supplementary Figures 3D,E**). These data suggest that the lower inflammation observed in FoxP3^{Cre}Tbx21$^{fl/fl}$ mice is unlikely due to an enhanced suppressive capacity of Tbet-deficient Tregs.

Conventional Th1 Cells Development Requires the Presence of Functional Th1-Like Tregs

In order to assess whether the phenotype shown by FoxP3^{Cre}TBX21$^{fl/fl}$ mice was caused by impaired Th1 cells in the absence of IFNγ-secreting Th1-like Tregs, LPMC from FoxP3^{Cre}TBX21$^{fl/fl}$ and FoxP3^{Cre}TBX21$^{wt/wt}$ mice were isolated

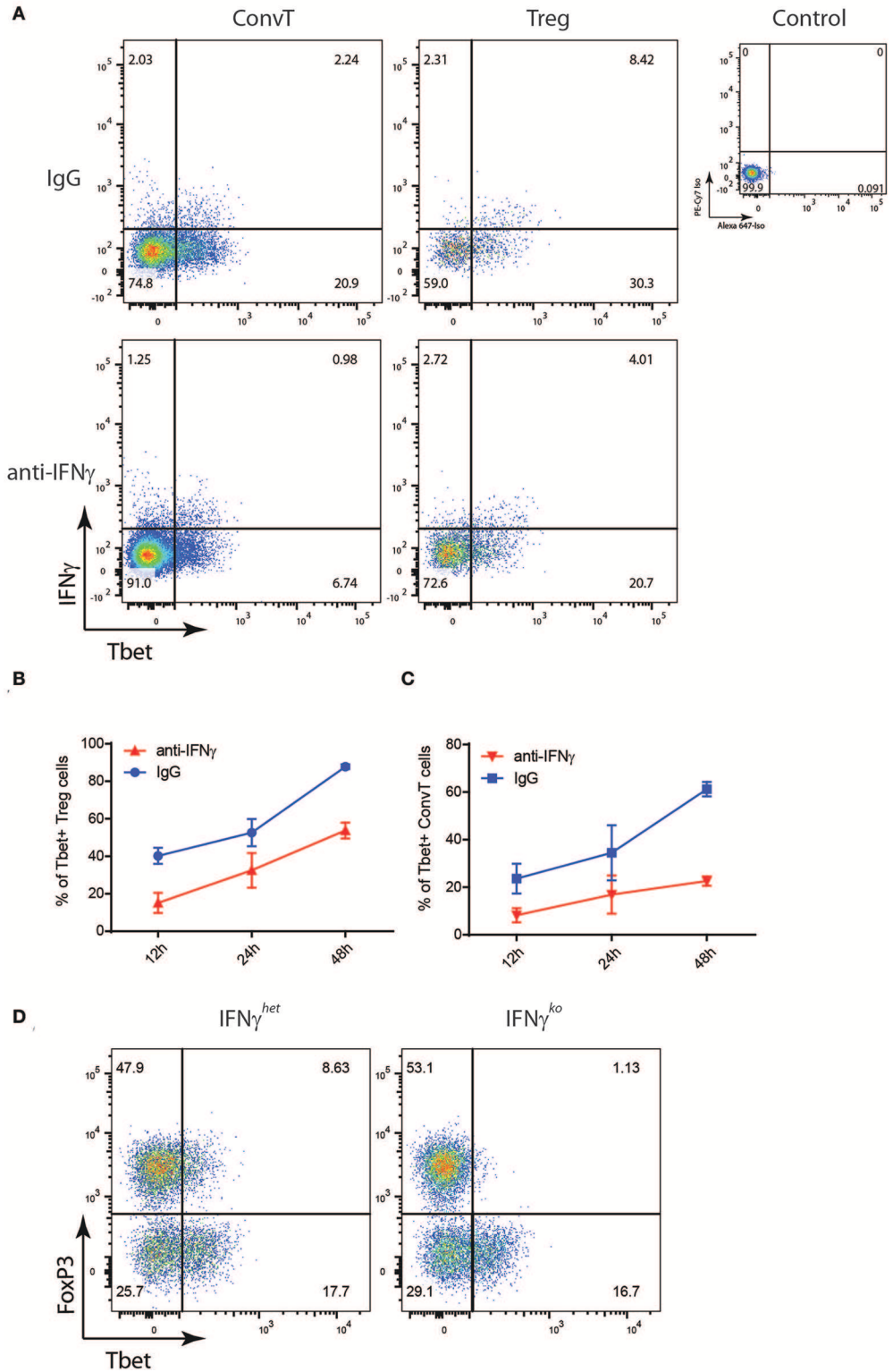

FIGURE 5 | Total CD3+CD4+ T cells from the spleen of reporter mice were polyclonally activated in the presence of neutralizing anti-IFNγ antibody or control IgG. **(A)** Representative dot plots showing Tbet and IFNγ expression on gated Treg and ConvT cells after 24 h of stimulation. Tbet and IFNγ isotype control stainings gating on CD4+ cells are shown (upper right plot). **(B,C)** Percent of Tbet+ cells among Treg **(B)** and ConvT **(C)** cells in the presence of anti-IFNγ or control IgG at different time points. Each point represents the mean ±SD of three experiment performed. **(D)** Representative dot plots showing FoxP3 and Tbet expression in LP CD3+CD4+ T cells of DSS-treated IFNγ^{ko} and IFNγ^{het} mice. Numbers in the dot plot quadrants represent the relative frequency of cell subpopulations.

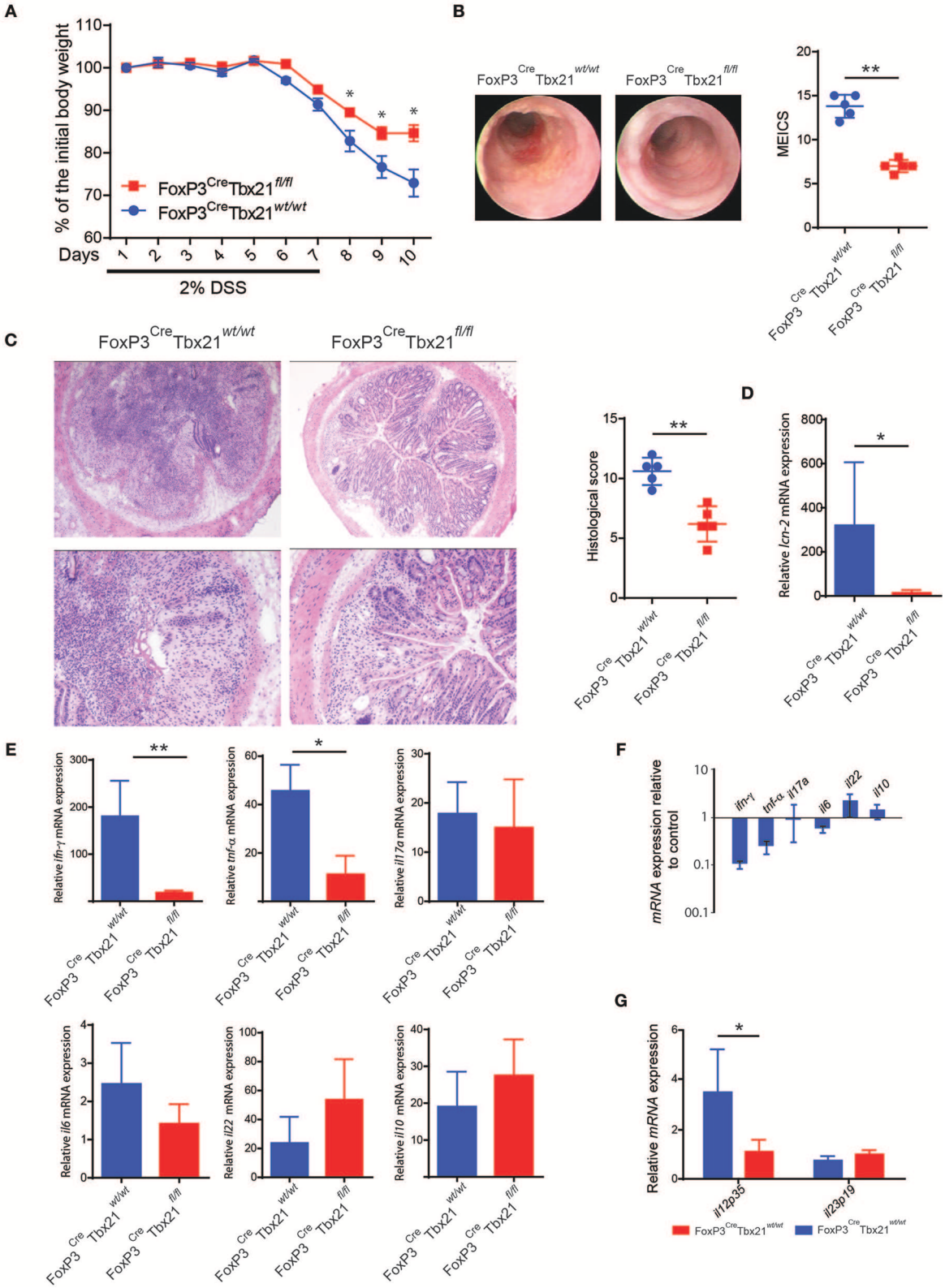

FIGURE 6 | (A) Body weight variation relative to the baseline of Treg-specific FoxP3 conditional knockout (FoxP3^{Cre}Tbx21$^{fl/fl}$) and control mice (FoxP3^{Cre}Tbx21$^{wt/wt}$) upon DSS treatment. Results from one representative experiment out of four performed is shown (mean percentage ±SEM). **(B)** Endoscopic pictures and endoscopic

(Continued)

FIGURE 6 | severity grading of knockout and control mice at the end of one representative experiment (Day 10). **(C)** Representative histological section from the colons of FoxP3 knockout and control mice at the end of the experiment and cumulative histologic severity scoring of the experiment shown in **(A)**. *lcn-2* **(D)** and cytokine **(E,G)** mRNA expression in the colon tissue of DSS-treated FoxP3 knockout and control mice. Vertical bars indicate the average ±SD analyzed in the pool of mice from four independent experiments. **(F)** Fold change of cytokine mRNA expression, as indicated, in FoxP3 knockout mice relative to control mice. $^{*}p < 0.05$; $^{**}p < 0.01$.

at the end of the DSS treatment and analyzed by flow cytometry. As expected, Tbet-expressing FoxP3+ cells were virtually absent in $FoxP3^{Cre}TBX21^{fl/fl}$ mice while about 20% of FoxP3+ cells from $FoxP3^{Cre}TBX21^{wt/wt}$ control mice co-expressed Tbet (**Figures 7A,B**). Interestingly, in TBX21 deficient mice, Tbet+ cells were also reduced by 3-fold among ConvT cells thus indicating that the expression of Tbet in Tregs is required for the development of Th1 cells in the inflamed colon. The expression of IFNγ in ConvT cells was also significantly reduced in the absence of Tbet-expressing Tregs (**Figures 7C,D**). The reduced expression of Tbet and IFNγ resulted to be specific for CD4+ ConvT cells since their expression remained unaffected in CD8+ cells from $FoxP3^{Cre}TBX21^{fl/fl}$ inflamed mice (**Supplementary Figures 4A,B**). Since LPMC from DSS-treated $FoxP3^{Cre}TBX21^{fl/fl}$ mice showed reduced expression of p35IL12, we wondered whether the defective induction of Tbet in ConvT cells in the presence of Tbet deficient Tregs observed occurred independently of innate immune cells. To this end, total CD4+ T cells isolated from the spleen of $FoxP3^{Cre}TBX21^{fl/fl}$ or $FoxP3^{Cre}TBX21^{wt/wt}$ control mice were polyclonally activated in the absence of antigen presenting cells. After 12 h, the induction of Tbet in ConvT cells from $FoxP3^{Cre}TBX21^{fl/fl}$ mice resulted suppressed as compared to controls (**Figures 8A,B**). In keeping with Tbet expression, IFNγ+ cells were significantly reduced in both Tregs and ConvT cells from $FoxP3^{Cre}TBX21^{fl/fl}$ mice as compared to $FoxP3^{Cre}TBX21^{wt/wt}$ cells (**Figure 8C**).

DISCUSSION

Tregs have been for long considered terminally differentiated, phenotypically stable cells characterized by suppressive capacity. Tregs are crucial for tolerance toward self and harmless antigens. However, the observation that inflammation can induce pro-inflammatory features in Tregs has challenged this concept. The phenotypic changes that may affect Tregs in different inflammatory conditions, are summarized by the terms "stability" and "plasticity." As for stability, it is meant the loss of FoxP3 expression and the subsequent acquisition of an effector phenotype (26–28). Although this phenomenon has been observed in animal models of immune mediated diseases, it might be circumscribed to a subset of not fully differentiated Tregs in which room for reprogramming in inflammatory conditions still exists (29). Nevertheless, Tregs have been shown to transiently or permanently express T-helper-master regulator transcription factors while maintaining FoxP3 expression. Though, the functional role of these phenotypical changes remains unclear. The expression of Tbet in Tregs has been shown to be required to efficiently control Th1 cells. Koch et al. demonstrated that the IFNγ released in the inflammatory environment induces Tbet in Tregs and that Tbet is required for the expression of the chemokine receptor CXCR3 to address Tregs at the site of inflammation where Th1 cells needs to be kept in check (13). However, in other conditions, the expression of Tbet in Tregs has been associated with the acquisition of a Th1-like pro-inflammatory phenotype characterized by the expression of IFNγ driven by IL12 signaling (14, 15).

The presence of Th1-like Tregs has been observed in different pathologic conditions such as multiple sclerosis (MS) (18), type 1 diabetes (T1DM) (17), autoimmune hepatitis after liver transplant (30), and in animal models of intestinal inflammation (19, 20). Although Tregs have been shown to accumulate in patients affected by IBD, a direct characterization of Th1-like Tregs in this pathologic condition and their functional role in intestinal inflammation is currently missing.

In this study we showed that active IBD is characterized by the accumulation of a sizable fraction of Tregs expressing both Tbet and IFNγ. Since Th1-like Tregs were upregulated in inflamed areas but not in patients with endoscopic disease remission or in uninflamed controls, we hypothesized that Th1-like Tregs could contribute to the inflammatory flares characterizing the relapsing-remitting inflammation observed in IBD. To address this issue, we investigated the role of Th1-like Tregs in the dextrane sodium sulfate (DSS) model of colitis. Similarly to human IBD, Th1-like Tregs accumulated in the inflamed colon. In this model, the upregulation of Tbet and IFNγ in Tregs preceded that of conventional CD4+ T cells (ConvT) and at day 10 the number of Th1-like Tregs represented one third of the total IFN-γ expressing CD4+ T cells. These findings are in agreement with the data reported by Feng et al. in the CBir-1 model of intestinal inflammation (19). In this model the transfer of colitogenic naïve CD4+ T cells specific for the immunodominant commensal antigen, CBir1 flagellin (CBir-1Tg) but not of naïve CD4+ T cells from OTII mice which do not induce colitis in immune-deficient $TCRb/d^{-/-}$ mice was associated to the generation of IFNγ-expressing Th1-like Tregs. Similarly, intestinal inflammation developing in TLR4- and IL10-deficient mice was accompanied by the accumulation of Th1-like Tregs. Thus, the induction of Th1-like Tregs characterizes the development of intestinal inflammation and it can be reproduced in different mouse models (20).

In agreement with previously published data, IFNγ was the main inducer of Tbet in Tregs both *in vitro* and *in vivo* (13–15). Indeed, the IFNγ neutralization in cell cultures of activated CD4+ T cells and the analysis of IFNγ deficient mice treated with DSS, demonstrated that IFNγ is required for the induction of Tbet in the lamina propria Tregs. In contrast to Tbet expression, IFNγ secretion by Tregs has been shown to be dependent on IL12 stimulation and intracellular STAT4 activation. Indeed, the

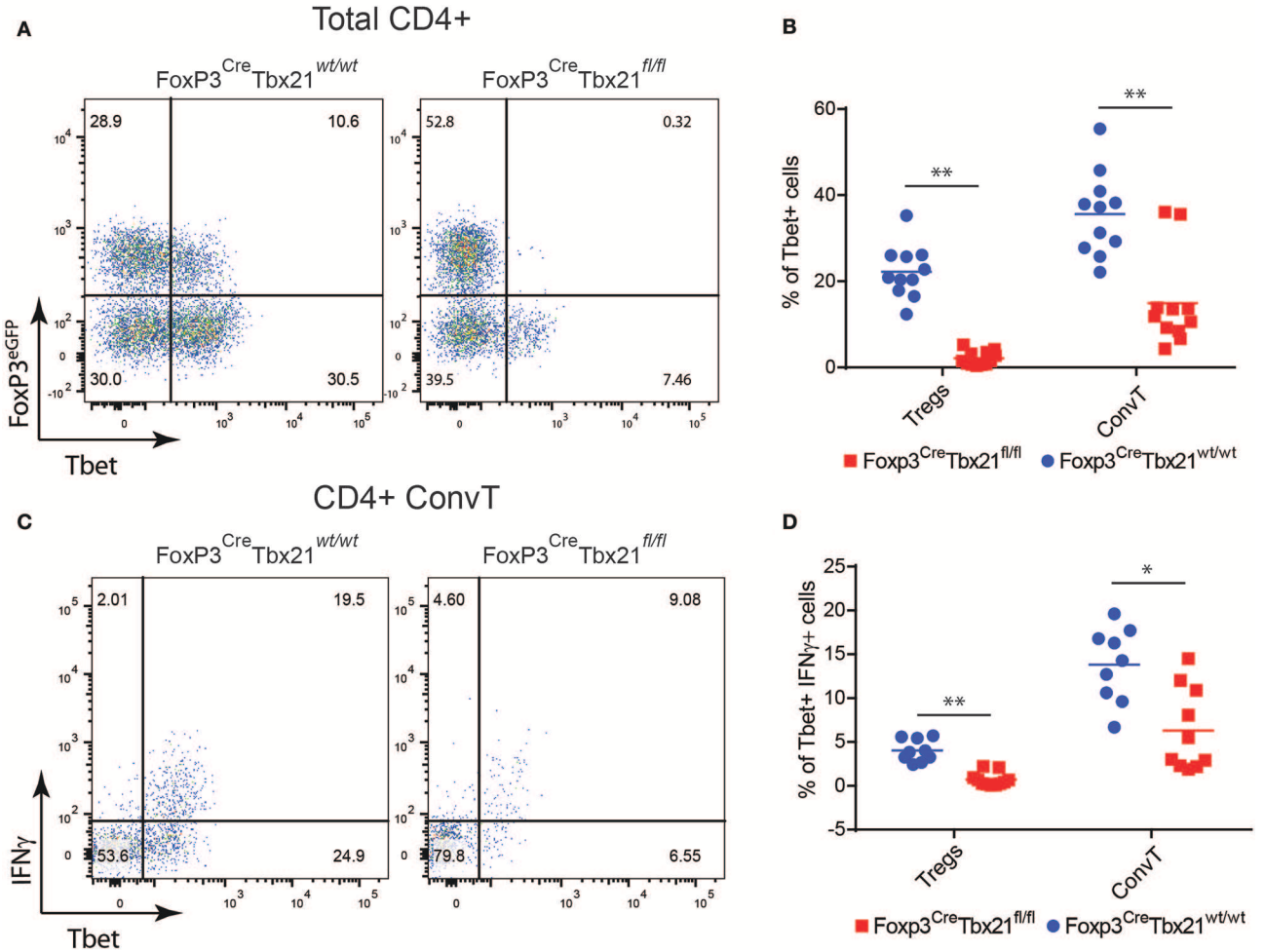

FIGURE 7 | (A) Representative dot plots showing FoxP3^{eGFP} and Tbet expression in LP CD3+CD4+ cells isolated from the colons of Treg-specific FoxP3 conditional knockout (FoxP3^{Cre}Tbx21$^{fl/fl}$) and control mice (FoxP3^{Cre}Tbx21$^{wt/wt}$). **(B)** Frequency of Tbet+ cells among LP Treg and ConvT cells from Tbet knock out and control mice. **(C)** Representative dot plots showing IFNγ and Tbet expression in LP ConvT cells isolated from Tbet knockout and control mice. **(D)** Frequency of Tbet+IFNγ cells among LP Treg and ConvT cells from Tbet knockout and control mice. Numbers in the dot plot quadrants represent the cell subpopulation relative frequency. Horizontal bars in **(B,D)** indicate the mean value and symbols represent each animal analyzed from three independent experiments. *$p < 0.05$; **$p < 0.01$.

prolonged expression of Tbet in Tregs induces the activation of the IL12 receptor locus in Tregs making these cells responsive to IL12 and inducing the expression of IFN-γ (15). In our *in vitro* system Tregs expressed IFNγ in the absence of IL12, thus indicating that Tbet expression is sufficient to induce the expression of IFNγ, not excluding the possible boosting effect of IL-12 in these cells. Functionally, Tbet expression in Tregs sustained intestinal inflammation since Treg-specific Tbet conditional knockout mice showed milder colitis after DSS treatment as compared to control mice. Tbet knockout mice were characterized by reduced expression of Th1- but not Th17-related cytokines. IL10, which has been shown to downregulate IFNγ expression and to protect from colitis was not increased in Tbet knockout mice (31). However, IL10 expression was not altered in the Tbet knockout Tregs as compared to the wild type controls. The phenotype of Tbet knockout mice was also unlikely due to increased Tregs suppressive capacity since Tbet-deficient Tregs were as suppressive as Tbet proficient Tregs both *in vitro* and *in vivo* in the adoptive transfer model of colitis. However, our data do not rule out the possibility that Tbet-expressing Tregs isolated from the inflamed gut might permanently or transiently lose their suppressive capacity further sustaining the development of inflammation. Finally, the reduced expression of IFNγ among lamina propria CD4+ T cells in DSS-treated knockout mice was not restricted to Tbet-deficient Tregs but it also involved conventional T cells, thus indicating that the Th1 immune response depends on the presence of Th1-like Tregs.

We exclude the possibility that the low Tbet expression in ConvT cells results from a transient activation of the Foxp3 locus during the differentiation of Th1 cells leading to the deletion of Tbet in these cells. Indeed, by using FoxP3 fate mapping reporter mice we were able to monitor the presence of T cells which

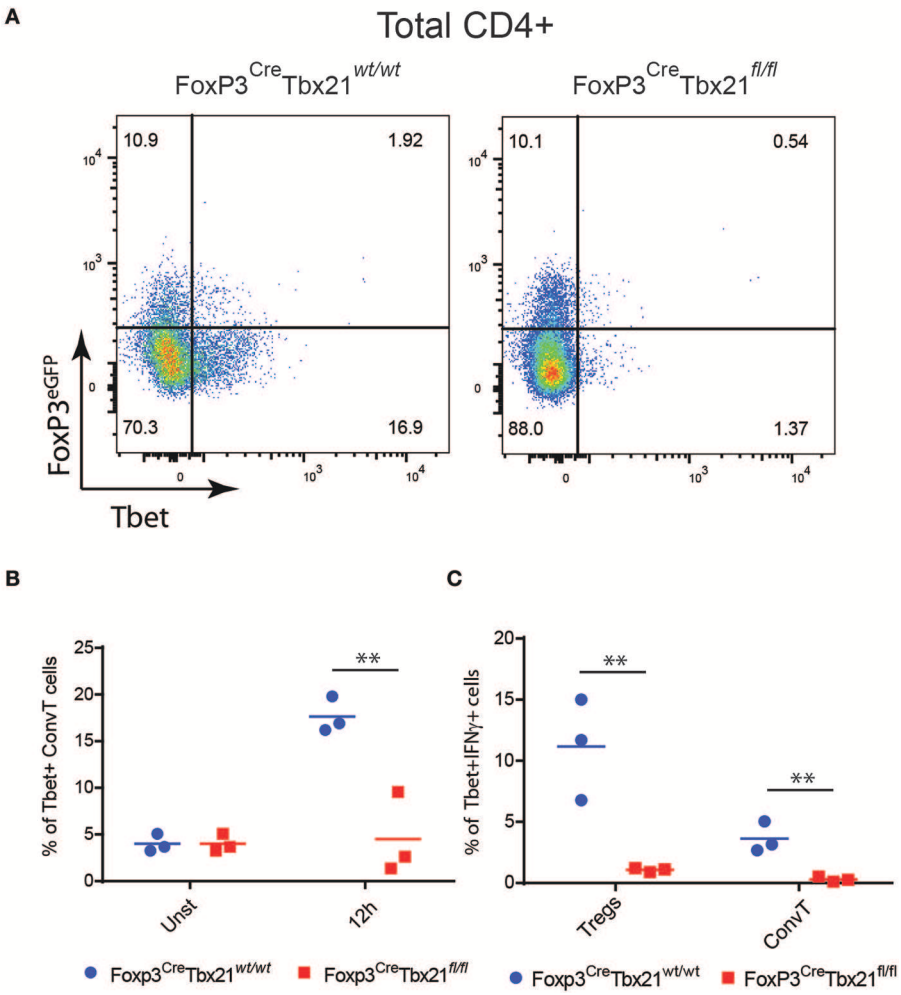

FIGURE 8 | (A) Representative dot plots showing the expression of FoxP3^{eGFP} and Tbet in CD3+CD4+ T cells from the spleen of Treg-specific Tbet conditional knockout (Foxp3^{Cre}Tbx21$^{fl/fl}$) or control mice (Foxp3^{Cre}Tbx21$^{wt/wt}$) polyclonally activated *in vitro* for 12 h. **(B)** Frequency of Tbet+ cells gated on ConvT cells of CD3+CD4+ T cells from Tbet knockout or control mice polyclonally activated *in vitro* for 12 h or left unstimulated. **(C)** Frequency of Tbet+IFNγ+ cells gated on Treg and ConvT cells of CD3+CD4+ Tcells knockout and control mice as in **(B)**. Numbers in the dot plot quadrants represent the relative frequency of cell subpopulations. Horizontal bars in **(B,C)** indicate the mean value of results obtained from three independent experiments. $^{**}p < 0.01$.

have transiently expressed FoxP3. In these mice the so called "ex FoxP3+ cells," marked by the permanent expression of the tdRFP fluorescence even in the absence of FoxP3 expression, represented a negligible fraction of CD4+ T cells and they were excluded from the analysis. Moreover, the frequency of ex-Tregs in Tbet knockout mice did not differ from controls thus indicating that Tbet expression in Tregs does not influence Treg phenotypic stability.

One possible interpretation of these results is that in the very initial phase of inflammation Th1-like Tregs operate as enhancer of the Th1 differentiation process. Different observations sustain this hypothesis. First of all, the relative increase of Tbet-expressing cells was higher among Tregs as compared to ConvT cells *in vitro* and *in vivo* after DSS treatment. Moreover, the induction of Tbet in Tregs preceded that of ConvT cells during DSS treatment when histologic signs of inflammation were barely detectable. Since the expression of Tbet by Tregs was dependent on IFNγ, the ready induction of Tbet in Tregs as compared to ConvT cells might be explained by the FoxP3-dependent upregulation of IFNγ receptor in Tregs vs. ConvT cells (32). Finally, the reduced expression of IFNγ observed in the lamina propria of conditional knockout mice was not limited to the absence of IFNγ-secreting Th1-like Tregs but it was associated to a more general impairment of the Th1 immune response.

The role of Th1-like Tregs as enhancer of Th1 immune response in the early phase of inflammation is also supported by the observation that miR146-deficient Tregs, which are characterized by the upregulation of STAT1-mediated intracellular signaling and IFNγ expression, increased the expression of Th1 cytokines in CD4+ T effector cells in a

bone marrow chimera system (32). Moreover, the expression of Tbet and IFNγ in Tregs contributed to the development of Th1-mediated lethal disease in a model of *T. gondii* infection (16).

Interestingly, and in agreement with our data, the upregulation of Tbet and IFNγ in Tregs induced by either mir146 deficiency or *T.Gondii* infection did not affect Treg suppressive activity thus confirming that the Th1 enhancer activity exerted by Tregs during inflammation is not associated with an impaired suppressive capacity.

The next fundamental question is how Th1-like cells enhance the Th1 immune response. It is possible that IFNγ expressed by Tregs directly contributes to the differentiation of conventional Th1 cells in a paracrine manner. However, IFNγ could also promote the differentiation of Th1 cells by upregulating the expression of IL12 in dendritic cells. Accordingly, IL12p35 but not IL23p19 was downregulated in the lamina propria of Tbet conditional knockout mice.

In conclusion, based on our data, we propose a model in which during the development of intestinal inflammatory flares, Th1-like Tregs enhances the initial phase of inflammation by promoting the development of Th1 cells. In this this context, Th1-like Tregs would represent an another source of IFNγ-producing cells involved in colitis development. Indeed, in addition to the classic Th1 differentiation from a naïve T cells, Th1 cells have been shown to derive from Th17 cells conversion in different inflammatory conditions including colitis. Therefore, the generation of Th1-like Tregs would represent just another moment of a general Th1-skewing process involving different T cell sub-types during the intestinal inflammation (33, 34).

At the same time, Tregs maintain their suppressive activity on effector cell proliferation thus avoiding an excessive inflammatory response. A dysregulation of this process, causing a lower threshold of activation of the Treg-mediated pro-inflammatory activity, might contribute to the generation of chronic inflammation in the gut and to the pathogenesis of IBD.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Tor Vergata University Hospital, Rome. Protocol number: 154/12. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Authorization No: 324/2006-PR issued by the Ministry of Health on 29/03/2016.

AUTHOR CONTRIBUTIONS

MD and AR contributed to the experimental design and performed the experiments. EF performed the experiments. FF, CS, and SO analyzed the lamina propria cells from human samples. FC collected the bioptic samples from IBD patients and reviewed the manuscript. AF analyzed the data. GM contributed to the study design. H-JF provided the Rosa26tdRFP reporter mice. MF designed the experiments, analyzed the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02158/full#supplementary-material

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MINI REVIEW
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Foxp3 Instability Helps tTregs Distinguish Self and Non-self

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Regulatory T cells (Tregs) are small subsets of CD4 T cells that play a central role in the controlling of immune tolerance. Tregs are either generated in the thymus (tTregs) or the periphery (pTregs), and both express the master transcription factor Foxp3. Stable expression of Foxp3 is important for the maintenance of Tregs identity and their suppressive function. Similar to conventional T cells, Tregs can recognize both self- and non-self-antigens, and TCR engagement leads to Treg activation and the generation of effector Tregs. Emerging shreds of evidence suggest Tregs are not always stable, even fully committed mature tTregs, and can lose foxp3 expression and programming to effector-like T cells. In this review, we summarize recent findings in Treg instability and the intrinsic and extrinsic mechanisms in controlling the Foxp3 expression. Finally, we propose a new hypothesis that Foxp3 instability might help tTregs distinguish between self and non-self-antigens.

Keywords: Treg, Foxp3, instability, self and non-self-discrimination, TCR

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INTRODUCTION

T cells are one of the major components of the adaptive immune system which protects against all kind of pathogens, harmful substances, and foreign tissues. Immature T cells expressing an enormous number of TCRs generated by random VDJ recombination undergo selection in the thymus, where self-reactive T cells are clonally deleted through negative selection. However, this mechanism of controlling self-reactive T cells, known as central tolerance, is not perfect. Some potentially autoreactive T cells escape deletion in the thymus and migrate to the periphery. In recent years, we have learned that suppression of autoreactive lymphocytes relies on a subset of T lymphocytes called regulatory T cells, a small subpopulation of $CD4^+$ T cells characterized by expression of the forkhead transcription factor Foxp3 (1).

Nishizuka and Sakakura were the first to show that thymus-derived Tregs mediate dominant self-tolerance (2). Their study showed that neonatal thymectomy around day 3 after birth resulted in severe autoimmune diseases, which could be prevented by adoptive transfer of thymocytes or splenocytes from adult euthymic mice (2). These observations demonstrated that a T cell subset generated in the mouse thymus after the third day of life could prevent autoimmunity. This T cell subset was later identified as a thymus-derived $CD25^+$ $CD4^+$ T cell population capable of protecting animals from autoimmune diseases (3, 4). However, CD25 is an activation marker, and by itself, is insufficient for identifying tolerance- and inflammation-promoting cells during an immune response. The breakthrough in the field came when the transcription factor Foxp3 was identified as the master regulator of Tregs (5–7). Expression of Foxp3 faithfully distinguishes naturally occurring thymic, as well as peripheral, $CD25^+CD4^+$ Tregs from naive $CD25^-CD4^+$ T cells or activated $CD4^+$ T cells. Moreover, sustained Foxp3 expression in mature Tregs is critical for maintaining of the Treg cell identity and suppression of life-threatening autoimmunity (8).

Although Tregs are likely to represent a stable cell lineage with regulatory functions, accumulating evidence suggests that Foxp3$^+$ Tregs retain plasticity and can be "reprogrammed" into T helper cells under certain environmental conditions (9–12). In this review, we summarize recent results on Treg instability and discuss their implications in distinguishing self and non-self.

MECHANISMS OF FOXP3 INDUCTION AND MAINTENANCE

The induction and maintenance of Foxp3 distinguish Tregs from the other T helper cell populations. Interestingly, these two processes are largely separable and regulated by Foxp3 promoter and three conserved non-coding sequences (CNS) located around the first intron and the first coding exon of the Foxp3 gene (13–15). Intrathymic differentiation of Tregs is synchronized with positive and/or negative selection and starts mostly at the CD4$^+$CD8$^-$ single-positive (CD4SP) stage (16, 17). By using TCR and antigen double transgenic systems, it was shown that CD25$^+$CD4$^+$ cells can differentiate into Tregs in the thymus when the cognate antigen is presented by thymic stromal cells (18, 19). These results suggest that Treg cells develop from CD4 SP T cells possessing TCRs with high avidity toward self-antigens (20, 21). Thymic Foxp3 induction and Treg lineage commitment are the synergistic effects of TCR signaling, co-stimulatory signals through CD28 and common γ-chain cytokine signals, particularly IL-2 signal (22–26). Transcription factors such as NFAT, AP-1, Nr4a factors, and STAT-5 can drive Foxp3 promoter activation in response to TCR and IL-2 signaling (27–29). Tregs can also be induced in the periphery from naïve conventional CD4$^+$ T cells, and these pTregs play an important role in maintaining intestinal mucosal immune tolerance and maternal-fetal tolerance (30–32). Interestingly, pTregs and tTregs have different dependence on CNS-1, which contains a TGF-β-NFAT response element, and is indispensable for peripheral, but not thymic, Foxp3 induction (15, 33). Additionally, CNS3, another cis-element regulating Foxp3 locus, acts as a pioneering element essential for inducing of Foxp3 expression (13). CNS3 recruits c-Rel and Foxo family transcription factors such as Foxo1 and Foxo3, which can open the Foxp3 gene locus, thereby facilitating Foxp3 induction (34, 35).

Stable expression of Foxp3 is dependent on CNS2, a CpG-rich region within Foxp3 locus. CNS2, also called Treg specific demethylation region (TSDR), is dispensable for Foxp3 induction but essential for heritable maintenance of Foxp3 expression in dividing Tregs (13, 14). CNS2 is fully methylated in conventional T cells (Tconvs) and highly methylated in Tregs generated recently in the thymus. Demethylation of CNS2 contributes to stable Foxp3 expression in Tregs (36, 37). The initiation of TSDR demethylation is Foxp3-independent, as the "wannabe" Tregs, which transcribe the Foxp3 locus but do not express Foxp3 protein, show demethylation of the TSDR (13, 36). Recent studies have revealed the roles of Ten-eleven translocation (TET) proteins, which can induce the demethylation of 5-methylcytosine (5mC) in a cell cycle-independent way, in the demethylation of CNS2 (38). By using CD4-Cre and Foxp3-Cre-mediated depletion of Tet2/Tet3, Rao et al. and Yoshimura et al. showed that the Tet proteins play a critical role in demethylating TSDR to ensure stable Foxp3 transcription (39, 40). Once the TSDR has been demethylated, Foxp3 protein, cooperating with other transcription factors such as Runx/Cbfβ, STAT5, CREB/ATF, and Ets-1, binds to the demethylated TSDR and stabilizes its own transcription through a positive feedback mechanism (13, 41–43). Rao et al. also observed that four CpGs in CNS1 and 11 CpGs in CNS2 share a similar CpG methylation pattern (38). Collectively, these findings show that the establishment of stable expression of Foxp3 occurs in a two-step process, the first step being the Tet- dependent demethylation of TSDR, followed by Foxp3-dependent self-enforcement. Moreover, the metabolic status during tTreg cell activation and environmental cytokine cues also contribute to the stability of Foxp3 expression (44–49).

EVIDENCE OF INSTABILITY OF FOXP3

Accumulating evidence suggests that Foxp3$^+$ cells are not terminally differentiated. Indeed, *in vitro* culture system and *in vivo* transfer experiments showed that a fraction of Tregs can lose their Foxp3 expression and acquire the ability to produce the corresponding Th cytokines depending on their microenvironment (10, 50, 51). To overcome the limitations of the relatively artificial experimental setting used in these experiments and directly address the problem *in vivo*, we have used genetic lineage-tracing approaches. To identify exFoxp3 T cells in the normal T cell repertoire, we generated a fate-mapping system by crossing ROSA26 YFP-reporter mice with Foxp3 bacterial artificial chromosome (BAC) transgenic mice expressing a GFP-Cre fusion protein (9). We found that 10–15% of YFP$^+$ cells did not express Foxp3 and GFP, and these GFP$^-$YFP$^+$ cells displayed an activated memory T cell phenotype with the ability to produce pro-inflammatory cytokines, IFN-γ and IL-17. Moreover, when crossed with transgenic mice expressing a diabetogenic TCR, the frequency of exFoxp3 cells increased in the inflamed pancreas and these cells conferred autoimmune diabetes upon adoptive transfer into lymphopenic mice. Similar results were observed by using MOG tetramer to identify antigen-specific Tregs in an experimental autoimmune encephalomyelitis (EAE) model, further supporting the notion that Tregs can be converted into pathogenic T helper cells *in vivo* (11). Collectively, these observations suggest that Foxp3$^+$ Tregs can lose Foxp3 expression and undergo lineage reprogramming in response to certain extrinsic cues such as lymphopenia and inflammation.

CONTRADICTION AND POSSIBLE EXPLANATIONS

Conclusions drawn from these studies have generated great debates. Treg plays a critical role in maintaining self-tolerance and many Tregs are biased toward self-recognition. In this context, unlimited functional reprogramming of Tregs to pathogenic effector T cells could have a disastrous effect on the host. The reprogramming model has been challenged by

a study from Rudensky et al., who utilized an inducible-labeling approach by knocking in a cDNA encoding GFP-Cre-ERT2 fusion protein into a 3′ untranslated region (UTR) of the Foxp3 gene, and then crossing these mice with ROSA26 YFP mice (52). When treated with tamoxifen to pulse label Foxp3$^+$ T cells, Rudensky et al. found that <5% of YFP$^+$ cells were Foxp3 negative. The frequency of Foxp3$^-$ cells did not increase even under inflammatory or lymphopenic conditions, suggesting that Foxp3 expression in Tregs was remarkably stable. The contradiction is unlikely to be due to an unfaithful reflection of endogenous Foxp3 expression by Foxp3-GFP-Cre BAC construct, as an independent Foxp3 GFP-Cre knock-in x ROSA26 RFP fate mapping system also showed that about 15% of peripheral Foxp3 traced RFP$^+$ T cells were indeed Foxp3$^-$ (53). Interestingly, different subsets of Tregs might have different Foxp3 stability, which even changes according to their developmental stages (33, 36, 37, 54). It is well-known that *in vitro* iTregs, induced by TGF-β, have highly methylated TSDR and are prone to lose Foxp3 (37). Rudensky et al. have shown that newly generated pTregs were unstable, about 50% of lineage-traced cells were Foxp3$^-$, whereas stable pTregs were generated only after 5 weeks upon transferring (33). The labeling efficiency of Tregs during fate mapping experiment, particularly the unstable pTregs, could be a potential factor for the inconsistent results among different models.

To resolve the controversies regarding the stability of Foxp3, Hori have proposed a heterogeneity model and postulated that exFoxp3s do not indicate real reprogramming of Tregs but reflect a minor population of uncommitted Foxp3$^+$ T cells which have lost their Foxp3 expression (55). According to this heterogeneity model, uncommitted Tregs are a minor fraction of the Foxp3$^+$CD25$^-$ subset, generated either from transient Foxp3 expression during the activation of peripheral T cells, or from immature tTregs that fail to demethylate CNS2 during thymic Treg development, thereby becoming susceptible to losing Foxp3 expression in lymphopenic and *in vitro* polarization settings (50). In contrast, most, if not all, of the CD25$^+$ Foxp3$^+$ T cells show stable Foxp3 expression under those conditions. Although the heterogeneity model is compatible with the two fate-mapping experiments mentioned before, it fails to shed any light on whether the fully committed tTregs can lose Foxp3 expression. Moreover, CD25 expression was significantly decreased following Treg activation and functional specialization (T-bet$^+$Treg, Bcl6$^+$TFR, etc.) (12, 56), and effector Tregs preferentially use ICOS instead of IL-2 signaling to support homeostasis and function (57). If so, can activated/effector Tregs maintain Foxp3 expression?

TCR SIGNAL DETERMINES THE INSTABILITY OF TREG

The heterogeneity and reprogramming models are not mutually exclusive. Recently, we have generated a new fate-mapping system which traces only the epigenetically stable tTregs, and that provides us a unique opportunity to address the above questions mentioned before (12). It has been demonstrated that the CNS1, which serves as a major TGF-β sensor, is critical for the generation of induced pTregs, but largely dispensable for tTreg development (33). Based on this observation, we developed a delta CNS1 Foxp3 BAC transgene mouse strain in which only

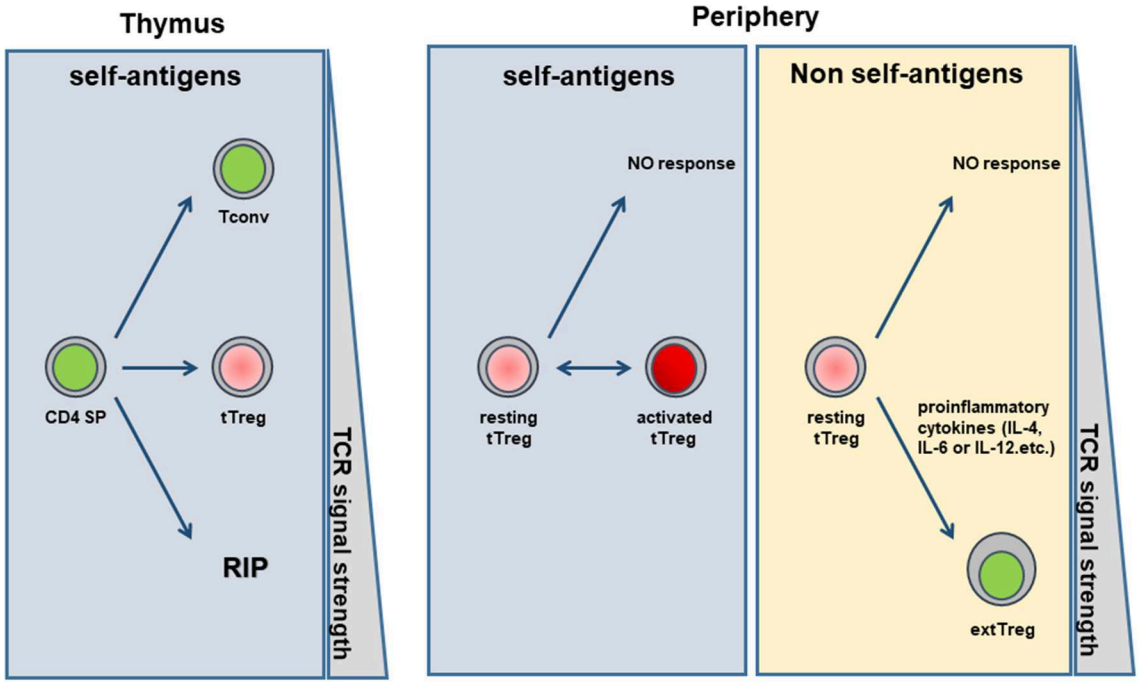

FIGURE 1 | Foxp3 instability helps tTregs distinguish between self and non-self-antigens.

tTregs express the Thy1.1-Cre fusion protein (referred to as Foxp3 delta CNS1-Cre-Thy1.1). Unexpectedly, the expression of Thy1.1-Cre reporter was significantly delayed in the thymus and marked the mature tTregs with hypomethylation of the TSDR. By using the Foxp3 delta CNS1-Cre-Thy1.1 x ROSA26 YFP fate-mapping system, we studied the stability of bona fide tTregs. We found that only ∼1% of mature tTregs lost Foxp3 expression in secondary lymphoid organs, indicating that tTregs are stable under homeostatic conditions. However, activation and sequential functional specialization of tTregs (conversion to T-bet$^+$Treg and Bcl6$^+$TFR) result in the loss of Foxp3 stability and reprogramming into T helper lineage. Destabilization of Foxp3 can also happen in Th2-like Treg or Th17-like Treg. Chatila et al. have shown that selective boosting of IL-4R signaling by introducing a gain-of-function IL-4Rα (Il4raF709) in Tregs can reprogram Treg to Th2 cells (58). Similarly, augmentation of Rorγt by knocking-out both T-bet and GATA3 in Tregs results in decreasing Foxp3 expression and generation of IL-17 producing cells (59). We further demonstrated that the signal switch from IL-2 to ICOS/PI3K during Treg activation account for Foxp3 instability and Treg reprogramming (12). Initiation of TSDR re-methylation is likely the key step for loss of Foxp3 expression (9), death of activated Treg, and survival and expansion of T helper cells could further drive the conversion.

To trace the functional specification of Tregs *in vivo*, we have used a dual lineage tracing mouse model in which the genetic tracing of Foxp3 and T-bet was simultaneously enabled (12). Interestingly, "exT-bet" Foxp3$^+$ cells (T-bet-tracer positive Tregs that had lost T-bet expression) reverted to resting-like Treg phenotypes with stable Foxp3 expression, whereas sustained T-bet expressing effector Tregs tend to lose Foxp3 expression. Together, these results suggest that over-stimulation likely promotes the instability of Tregs and converts them from immune-suppressing cells to immune-boosting cells.

FOXP3 INSTABILITY MAY HELP TTREGS DISTINGUISH SELF AND NON-SELF

Considering all these observations, we propose the hypothesis that Foxp3 instability may help tTregs distinguish between self and non-self-antigens (**Figure 1**). Both conventional T cells and tTreg develop in the thymus, possibly from the same pool of diversified immature T cells. Thymocytes are educated by an elaborate process, during which their fate was determined by the affinity of the TCRs for self-peptide-MHC complexes on APCs. CD4 SP cells that bind with high affinity to self-antigens undergo clonal deletion to limit autoimmunity, whereas the thymocytes that bind with low affinity against the self-ligands can survive and emigrate to the periphery as conventional T cells. Tregs are positively selected from the TCRs with the affinity between the clonally deleted autoreactive T cells and Tconvs. In the periphery, Tconvs usually remain inactive due to their low affinity to self-ligands. When the organism is infected by pathogens, Tconvs, having high affinity against foreign antigens, undergo clonal expansion to differentiate into effector cells and protect the host. Similar to T conventional cells, TCR engagement is also a critical step for Treg activation and gain of potent suppressive function (60, 61). Although tTregs are selected by self-antigens in the thymus, many studies have suggested that a substantial proportion of thymic Tregs recognize foreign antigens. Because TCRs on Tregs has an intermediate affinity to self-ligands, most of the self-ligands in the periphery can only activate tTregs to a certain extent, thereby maintaining a perfect window in which Treg activation is triggered but their stability is not impaired. In contrast to self-antigens, foreign antigens could trigger strong TCR signals due to lack of negative selection, and the strong signal could induce a high level of ICOS/PI3K activation that is detrimental to Treg suppressive activity and stability (12, 45, 62). In addition to TCR signaling, proinflammatory cytokines induced by infection such as IL-6, IL-12, or IL-4 can also have a disruptive effect on Treg stability (50, 51, 63). Thus, those Tregs, which respond vigorously are prone to losing their suppressive function, program into an immune-boosting cell, and contribute to clearance of pathogens. Indeed, loss of Treg stability has been observed in many pathologic conditions in response to foreign antigens such as infection and allergy (64–66). This hypothesis would explain many general phenomena in which Tregs can control weak immune responses, while being relatively incompetent to suppress strong immune responses, such as allo-skin grafts. Consistent with this notion, Amigorena et al. have demonstrated that Tregs could down-regulate low-avidity CD8 reactions but promote the high-avidity of CD8$^+$ T cell responses to foreign antigen (67).

CONCLUSIONS

Although many controversies remain, more and more pieces of evidence have supported the notion that Tregs can lose Foxp3 expression under certain conditions. In the past 10 years, the extracellular and intracellular signals that maintain or destabilize Foxp3 have been intensively investigated, however, the physiologic function of Treg instability is not fully understood. We propose a hypothesis that Foxp3 instability helps tTreg to distinguish self and non-self-antigens. A better understanding of this question might have important therapeutic applications in a variety of diseases ranging from tumor to infectious disease.

AUTHOR CONTRIBUTIONS

ZZ and XZ wrote the manuscript.

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Dynamic Imprinting of the Treg Cell-Specific Epigenetic Signature in Developing Thymic Regulatory T Cells

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Regulatory T (Treg) cells mainly develop within the thymus and arise from $CD25^+Foxp3^-$ ($CD25^+$ TregP) or $CD25^-Foxp3^+$ ($Foxp3^+$ TregP) Treg cell precursors resulting in Treg cells harboring distinct transcriptomic profiles and complementary T cell receptor repertoires. The stable and long-term expression of Foxp3 in Treg cells and their stable suppressive phenotype are controlled by the demethylation of Treg cell-specific epigenetic signature genes including an evolutionarily conserved CpG-rich element within the *Foxp3* locus, the Treg-specific demethylated region (TSDR). Here we analyzed the dynamics of the imprinting of the Treg cell-specific epigenetic signature genes in thymic Treg cells. We could demonstrate that $CD25^+Foxp3^+$ Treg cells show a progressive demethylation of most signature genes during maturation within the thymus. Interestingly, a partial demethylation of several Treg cell-specific epigenetic signature genes was already observed in $Foxp3^+$ TregP but not in $CD25^+$ TregP. Furthermore, $Foxp3^+$ TregP were very transient in nature and arose at a more mature developmental stage when compared to $CD25^+$ TregP. When the two Treg cell precursors were cultured in presence of IL-2, a factor known to be critical for thymic Treg cell development, we observed a major impact of IL-2 on the demethylation of the TSDR with a more pronounced effect on $Foxp3^+$ TregP. Together, these results suggest that the establishment of the Treg cell-specific hypomethylation pattern is a continuous process throughout thymic Treg cell development and that the two known Treg cell precursors display distinct dynamics for the imprinting of the Treg cell-specific epigenetic signature genes.

Keywords: Treg cell, Treg cell precursors, demethylation, epigenetic signature, IL-2, thymus, TSDR, Foxp3

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INTRODUCTION

$CD4^+$ regulatory T (Treg) cells are crucial for the maintenance of self-tolerance. The continuous expression of the lineage-specification factor, Foxp3, endows these immunoregulatory cells with long-term stability and suppressive activity (1, 2). Accordingly, mutations in the *Foxp3* locus can result in an autoimmune and inflammatory syndrome in mice and humans [Scurfy and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, respectively]

(3–5). Although the induction and maintenance of Foxp3 expression are crucial for the lineage identity and functionality of Treg cells, Foxp3 expression as such is not sufficient to ensure complete Treg cell phenotypic and functional properties. For instance, retrovirally induced ectopic expression of Foxp3 in $CD4^+CD25^-$ conventional T cells could not induce the complete set of Treg cell-specific signature genes (6, 7). In line with this, *in vivo* disruption of the *Foxp3* gene by green fluorescent protein (GFP; *Foxp3*^{gfpko} mice) resulted in $Foxp3^-GFP^+$ cells still expressing several Treg cell-specific signature genes (8). To this end, it was shown that the CpG DNA demethylation at a set of Treg cell-specific epigenetic signature genes essentially but independently complements Foxp3 expression for entire Treg cell functionality and long-term lineage stability (9–12). Although significant progress has been made in understanding the importance of epigenetic imprinting on generating stable Treg cells, factors that initiate and drive this imprinting process are still incompletely understood.

Induction of Foxp3 expression and acquisition of the Treg cell-specific CpG hypomethylation pattern take place during thymic Treg cell development. It is assumed that the majority (∼80%) of the Treg cell population originates from the thymus, termed thymus-derived Treg (tTreg) cells (13). The concurrent stimulation of the T cell receptor (TCR) and CD28 is viewed as the first step in a two-step model of thymic Treg cell development (14, 15). This model proposes that the first step is instructive for the up-regulation of the IL-2Rα subunit (CD25), resulting in the development of $CD25^+Foxp3^-$ Treg cell precursors ($CD25^+$ TregP). Due to the expression of the high affinity IL-2 receptor, these cells are supremely sensitive to IL-2 and, at least a part of this precursor population can differentiate into $CD25^+Foxp3^+$ Treg cells in a second step upon stimulation with IL-2 without further need for TCR-derived signals (15). Accordingly, IL-2- or CD25-deficient mice display impaired tTreg cell development, exhibiting ∼50% of normal Treg cell numbers among CD4 single-positive (SP) thymocytes (16, 17), and develop lymphoproliferative disease. Whether IL-2 signaling in $CD25^+$ TregP is sufficient to drive epigenetic imprinting characteristic of mature tTreg cells is, however, not known. In addition to this model of Treg cell development, other studies indicate that Treg cells can also arise from $CD25^-Foxp3^+$ Treg cell precursors ($Foxp3^+$ TregP) (18). Thus, it was proposed that TCR-CD28 co-stimulation and/or IL-15 might lead to the up-regulation of Foxp3 expression in CD4SP thymocytes (18, 19). Interestingly, Foxp3 was reported to be proapoptotic, and unless it is counterbalanced by IL-2 signals, $Foxp3^+$ TregP undergo apoptosis (18). NF-κB is essential for the generation of both precursor populations. While IkB_{NS} and c-Rel together control the induction of Foxp3 expression in $CD25^+$ TregP and $Foxp3^+$ TregP, it was shown that c-Rel supports the induction of CD25 in both precursors (20–22). Recently, Owen et al. reported that $CD25^+$ TregP and $Foxp3^+$ TregP contribute almost equally to the generation of mature tTreg cells, despite showing distinct maturation kinetics and cytokine responsiveness. Additionally, the mature tTreg cells derived from the two precursors differed in their transcriptomes, their interactions with self-antigens and their TCR repertoire (23). In line with this, $Foxp3^+$ TregP were shown to already possess a partially demethylated Treg-specific demethylated region (TSDR), while $CD25^+$ TregP exhibited a completely methylated TSDR comparable to $Foxp3^-$ CD4SP thymocytes (24). However, the dynamics of the imprinting and the establishment of the Treg cell-specific hypomethylation pattern as well as the involvement of IL-2 in the demethylation of the TSDR and other Treg cell-specific epigenetic signature genes in both precursor populations have not been investigated yet.

In this study, we assessed the dynamics of the imprinting of the Treg cell-specific epigenetic signature genes in tTreg cells and could demonstrate that $CD25^+Foxp3^+$ Treg cells show a progressive demethylation of most signature genes while maturing within the thymus. The two Treg cell precursors displayed distinct dynamics for the imprinting of the Treg cell-specific epigenetic signature genes, with $Foxp3^+$ TregP already showing a partially established Treg cell-specific hypomethylation pattern. Intriguingly, we found that IL-2 mainly impacts the establishment and progression of TSDR demethylation with a more pronounced effect on $Foxp3^+$ TregP when compared to $CD25^+$ TregP. Thus, the two thymic Treg cell precursors differ substantially in the establishment of the Treg cell-specific hypomethylation pattern.

MATERIALS AND METHODS

Mice

B6.*Foxp3*$^{tm1(CD2/CD52)Shori}$ ($Foxp3^{hCD2}$ reporter mice on C57BL/6 background), B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ.*Foxp3*$^{tm1(CD2/CD52)Shori}$ (CD45.1 congenic $Foxp3^{hCD2}$ reporter mice on C57BL/6 background) and B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ.*Foxp3*$^{tm1(CD2/CD52)Shori}$-*Rag1*$^{tm1(GFP)Imku}$ mice (CD45.1 congenic $Foxp3^{hCD2}$x$Rag1^{GFP}$ reporter mice on C57BL/6 background) (25, 26) were bred and maintained at the central animal facility of the Helmholtz Center for Infection Research (HZI, Braunschweig, Germany), which provides state-of-the-art laboratory animal care and service. B6.*Foxp3*$^{tm2.1(EGFP/cre)Shori}$.*Gt(ROSA)26Sor*^{tm1Hjf} mice ($Foxp3^{eGFPCre}$x$ROSA26^{RFP}$ fate-mapping mice on C57BL/6 background) (26) were bread and maintained at the animal facility of the RIKEN Center for Integrative Medical Sciences (Yokohama, Japan). All mice were housed in barriers under specific pathogen-free (SPF) conditions in isolated, ventilated cages, and handled by personnel appropriately trained as well as dedicated animal care staff to assure the highest possible hygienic standards and animal welfare in compliance with German, European and Japanese animal welfare guidelines. According to the German Animal Welfare Act (§4, section Discussion) sacrifizing animals solely to remove organs for scientific purposes is notified to the competent authority. This study was carried out in accordance with the principles of the Basel Declaration as well as recommendations as defined by FELASA (Federation of European Laboratory Animal Science Associations), the German animal welfare body GV-SOLAS (Society for Laboratory Animal Science), and the Institutional Animal Care at RIKEN using approved protocols. All mice were used at the age of 4–9 weeks.

Antibodies and Flow Cytometry

Cell suspensions were labeled directly with the following fluorochrome-conjugated anti-mouse antibodies purchased from

either BioLegend or eBioscience: CD4 (RM4-5), CD8α (53–6.7), CD24 (M1/69), CD25 (PC61.5), CD73 (TY/11.8), and anti-human CD2 (RPA-2.10). To block the Fc-receptors, the staining mix always contained unconjugated anti-FcRγIII/II antibody (BioXcell; final concentration 10 $\mu g\ ml^{-1}$). For exclusion of dead cells, 4′,6-Diamidine-2′-phenylindole dihydrochloride (Merck) was used. Stained cells were assessed by LSRFortessa™ or FACSCanto™ II flow cytometer (BD Biosciences) and data were analyzed with FlowJo® software (TreeStar).

Cell Sorting

Single-cell suspensions from thymi were depleted of APC-labeled $CD8\alpha^+$ cells using anti-APC microbeads (Miltenyi Biotec) and the autoMACS® Pro Separator (Miltenyi Biotec). The negative fraction was stained with fluorochrome-conjugated anti-mouse antibodies and subsets of CD4SP thymocytes were sorted using a FACSAria™ or a FACSAria™ Fusion (BD Biosciences).

Cell Culture

Sorted CD4SP thymocyte subsets were cultured at 37°C and 5% CO_2 for 3 or 5 days. For this purpose, 5×10^4 cells/well were placed within 100 µl of Roswell Park Memorial Institute medium (RPMI, Gibco) completed with 10% FCS, 50 U ml^{-1} penicillin, 50 U ml^{-1} streptomycin, 25 mM HEPES, 1 mM sodium pyruvate (all Biochrom AG), 50 µM β-mercaptoethanol (Gibco) containing 50 ng ml^{-1} recombinant mouse IL-2 (R&D Systems) into a round-bottom 96-well plate (Sarstedt).

DNA Methylation Analysis

Genomic DNA was isolated from sorted CD4SP thymocyte subsets using the DNeasy® Blood & Tissue Kit (Qiagen) and concentrated using the DNA Clean & Concentrator Kit (Zymo Research), both following the manufacturers' protocols. The DNA concentration was quantified with a Nanodrop 1000 spectrophotometer (Peqlab). Methylation analysis of the TSDR and other Treg cell-specific epigenetic signature genes was performed using bisulfite sequencing as described before (27). Exclusively, cells from male mice were used for the methylation analysis. For each CD4SP thymocyte subset, cells were pooled from 4 to 6 independent experiments to reach sufficient cell numbers for the methylation analyses.

Statistical Analysis

The GraphPad Prism software v7.0 (GraphPad) was used to perform all statistical analyses. Data are presented as mean ± standard deviation (SD). For comparison of unmatched groups, two-tailed Mann-Whitney test was applied and the *p*-values were calculated with long-rank test (Mantel-Cox). If comparing more than two groups Kruskal-Wallis-Test with Dunn's test was used. A *p*-value below 0.05 was considered as significant ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant).

RESULTS

Newly Generated Treg Cells Progressively Mature Within the Thymus

We had previously demonstrated that tTreg cell maturation, which manifests as a progressive demethylation of the TSDR, is a continuous process that progresses after up-regulation of Foxp3 expression (12). In order to study the dynamics of this maturation process in more detail and in a more precise system, we here made use of transgenic $Foxp3^{hCD2}xRag1^{GFP}$ reporter mice, which express green fluorescent protein (GFP) under control of the recombination-activating gene 1 (*Rag1*) promoter (25). In these mice, GFP expression identifies newly generated thymocytes and discriminates them from older and/or re-circulating T cells (28, 29). This is of specific importance as Treg cells were recently shown to re-enter the thymus from the periphery (30), thereby blurring the analysis of tTreg cell development. Here, we first confirmed by flow cytometric analysis that a decrease in CD24 expression, which is associated with thymocyte maturation (12, 31), correlates with a decrease in GFP intensity within the newly generated (Rag^{GFP+}) CD4SP thymocyte compartment (**Figure S1**). Futhermore, flow cytometric analysis of $CD24^{hi}$, $CD24^{int}$ and $CD24^{low}$ subsets among newly generated (Rag^{GFP+}) $CD25^+Foxp3^+$ Treg cells (**Figure 1A**) revealed a significant increase in CD25 expression levels as well as a trend-wise increase in $Foxp3^{hCD2}$ expression from the $CD24^{hi}$ to the $CD24^{int}$ Treg cell subset (**Figures 1B,C**). In contrast, no difference was observed in expression levels of CD25 and $Foxp3^{hCD2}$ between $CD24^{int}$ and $CD24^{low}$ Treg cell subsets.

The demethylation of Treg cell-specific epigenetic signature genes is a prerequisite for the stable and long-term expression of Foxp3 in Treg cells and their stable suppressive phenotype. This process was reported to be induced already at early stages of Treg cell development within the thymus (11). Additionally, progressive demethylation of the TSDR occurs along maturation of bulk $Foxp3^+$ CD4SP thymocytes (12). To gain a more precise insight into the dynamic imprinting of the Treg cell-specific hypomethylation pattern during thymic Treg cell maturation, we FACS-sorted $CD24^{hi}$, $CD24^{int}$, and $CD24^{low}$ subsets of newly generated (Rag^{GFP+}) $CD25^+Foxp3^{hCD2+}$ Treg cells. Subsequently, genomic DNA from these subsets was bisulfite treated and analyzed by pyrosequencing. Interestingly, we observed a successive demethylation of the TSDR, *Eos*, *Ctla-4*, and *Gitr* correlating with CD24 down-regulation (**Figures 1D,E**). Intriguingly, *Helios* was already partially demethylated in the $CD24^{hi}$ subset, and no further decrease in its methylation status during Treg cell maturation was observed (**Figures 1D,E**). Thus, these data suggest that newly generated (Rag^{GFP+}) $CD25^+Foxp3^{hCD2+}$ Treg cells continuously mature within the thymus, observed as increase in CD25 and $Foxp3^{hCD2}$ expression and progressive demethylation of distinct Treg cell-specific epigenetic signature genes.

$Foxp3^+$ TregP Arise at a More Mature Developmental Stage Than $CD25^+$ TregP and Display a Partially Demethylated Treg Cell-Specific Epigenetic Signature

After having shown that newly generated immature $CD24^{hi}CD25^+Foxp3^{hCD2+}$ Treg cells are already partially demethylated at several Treg cell-specific epigenetic signature genes, we next aimed to assess the methylation status of these signature genes at earlier developmental stages of Treg

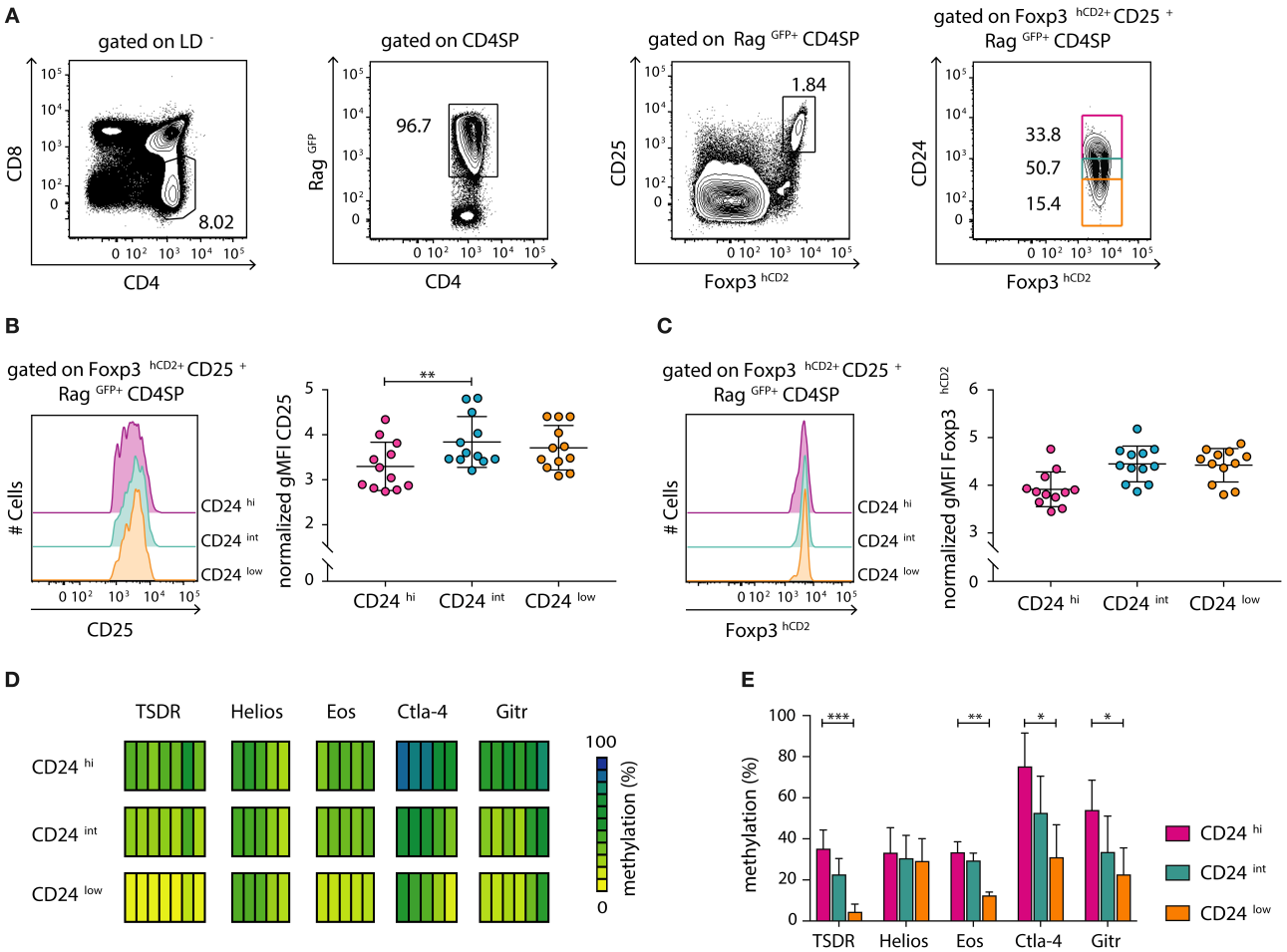

FIGURE 1 | Newly generated CD25^{+}Foxp3^{hCD2+} Treg cells progressively mature within the thymus. Thymocytes were isolated from Foxp3^{hCD2}xRag1^{GFP} reporter mice and analyzed by flow cytometry or prepared for subsequent methylation analysis. **(A)** Representative dot plots show the gating of CD4SP thymocytes among living cells (LD^{-}, LIVE/DEAD^{-}), newly generated Rag^{GFP+} cells among CD4SP thymocytes, CD25^{+}Foxp3^{hCD2+} Treg cells among Rag^{GFP+}CD4SP thymocytes, and CD24$^{hi/int/low}$ cells among CD25^{+}Foxp3^{hCD2+}Rag^{GFP+}CD4SP thymocytes. Numbers specify frequencies of cells in indicated gates. **(B,C)** (Left) Representative histograms depict the expression of CD25 **(B)** and Foxp3^{hCD2} **(C)** among CD24$^{hi/int/low}$ subsets of CD25^{+}Foxp3^{hCD2+}Rag^{GFP+}CD4SP thymocytes. (Right) Scatter plots summarize the data from three independent experiments and bars indicate mean ± SD. Each symbol represents an individual mouse. The significance was calculated using Kruskal-Wallis with Dunn's test (**$p < 0.01$). Data are given relative to the maximal span (x_{max}-x_{min}) per experiment. **(D,E)** Genomic DNA was isolated from sorted CD24$^{hi/int/low}$ subsets of CD25^{+}Foxp3^{hCD2+}Rag^{GFP+}CD4SP thymocytes for analysis of the methylation status of TSDR, *Helios*, *Eos*, *Ctla-4*, and *Gitr*. Cells were pooled from four independent sorts. **(D)** Each bar represents an individual CpG motif. Percentage of methylation is color-coded according to the scale. **(E)** Bar graph summarizes changes in the CpG methylation ratio of the Treg cell-specific epigenetic signature genes in CD24$^{hi/int/low}$ subsets of CD25^{+}Foxp3^{hCD2+}Rag^{GFP+}CD4SP thymocytes (bars indicate mean ± SD of all CpG motifs). The significance was calculated using Kruskal-Wallis with Dunn's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

cells. It was previously reported that tTreg cells arise from either CD25^{+}Foxp3^{-} (CD25^{+} TregP) or CD25^{-}Foxp3^{+} (Foxp3^{+} TregP) Treg cell precursors (15, 18, 23). Here, we first investigated the maturity of these two different Treg cell precursors and to this end analyzed the expression of CD24 among CD25^{+} TregP and Foxp3^{+} TregP in comparison to newly generated (Rag^{GFP+}) CD25^{+}Foxp3^{+} Treg cells by flow cytometry (**Figures 2A,B**). While CD25^{+} TregP displayed a rather immature phenotype with very high CD24 expression levels, Foxp3^{+} TregP showed a significantly decreased CD24 expression almost reaching the levels of CD25^{+}Foxp3^{+} Treg cells (**Figure 2B**). Accordingly, the majority of CD25^{+} TregP was found among CD24^{hi}Rag^{GFP+} CD4SP thymocytes, while Foxp3^{+} TregP were mainly enriched in the corresponding CD24^{low} subset (**Figure S2**). We also analyzed the maturity of Foxp3^{+} TregP in Foxp3^{GFPCre}xROSA26^{RFP} mice. In these fate-mapping mice, RFP expression labels Foxp3^{+} cells and their progeny, independently of continuous Foxp3 expression (26). We here found that Foxp3^{+} TregP are mainly constituted of RFP$^{-/low}$ cells when compared to CD25^{+}Foxp3^{+} Treg cells, whose majority has accumulated high levels of RFP (**Figure 2C**). Yet, also a small fraction of Foxp3^{+} TregP expressed high levels of RFP. These cells likely represent the previously described CD25^{-}Foxp3^{+} Treg cells re-entering the thymus from the

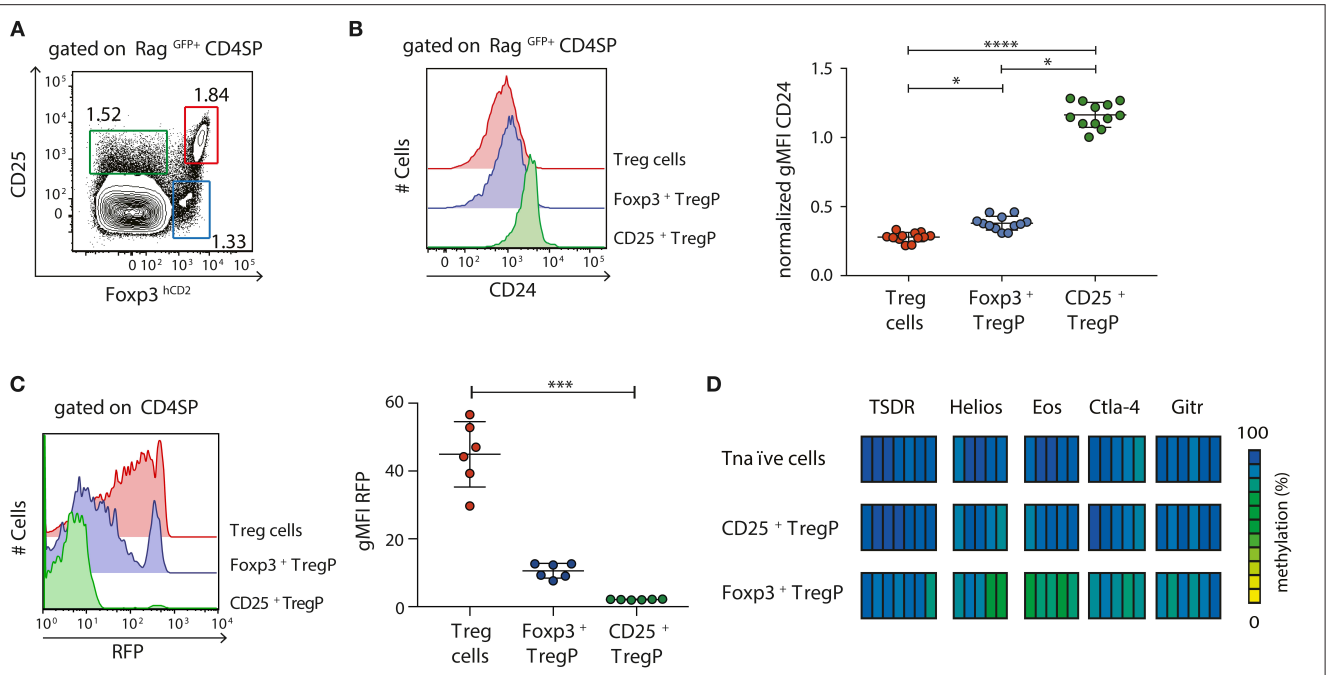

FIGURE 2 | Foxp3^{+} TregP already display a partially demethylated Treg cell-specific epigenetic signature. **(A,B)** Thymocytes were isolated from Foxp3^{hCD2}xRag1^{GFP} reporter mice and analyzed by flow cytometry. **(A)** Representative dot plot shows the gating of CD25^{+}Foxp3^{hCD2-} (CD25^{+} TregP), CD25^{-}Foxp3^{hCD2+} (Foxp3^{+} TregP), and CD25^{+}Foxp3^{hCD2+} (Treg cells) cells among Rag^{GFP+}CD4SP thymocytes. Numbers specify frequencies of cells in indicated gates. **(B)** (Left) Representative histograms depict CD24 expression among CD25^{+} TregP, Foxp3^{+} TregP and Treg cells. (Right) Scatter plot summarizes the data from three independent experiments and bars indicate mean ± SD. Each symbol represents an individual mouse. The significance was calculated using Kruskal-Wallis with Dunn's test (*$p < 0.05$; ****$p < 0.0001$). Data are given relative to the maximal span (x_{max}-x_{min}) per experiment. **(C)** Thymocytes were isolated from Foxp3^{GFPCre}xROSA26^{RFP} fate-mapping mice and analyzed by flow cytometry. (Left) Representative histograms depict RFP expression among CD25^{+} TregP, Foxp3^{+} TregP and Treg cells. (Right) Scatter plot summarizes the data from three independent experiments and bars indicate mean ± SD. Each symbol represents an individual mouse. The significance was calculated using Kruskal-Wallis with Dunn's test (***$p < 0.001$). **(D)** Thymocytes were isolated from Foxp3^{hCD2} reporter mice, and indicated populations pre-gated on newly generated CD73^{low}CD4SP thymocytes were sorted. Genomic DNA was isolated from sorted cells for analysis of the methylation status of TSDR, *Helios*, *Eos, Ctla-4*, and *Gitr*. Cells were pooled from two independent sorts. Each bar represents an individual CpG motif. Percentage of methylation is color-coded according to the scale.

periphery (28). Thus, Foxp3^{+} TregP newly developing in the thymus display an only transient developmental stage precluding accumulation of RFP.

Next, we assessed the methylation pattern of the Treg cell-specific epigenetic signature genes within the two different Treg cell precursors. Pyrosequencing was performed on bisulfite-treated genomic DNA from CD25^{+} TregP and Foxp3^{+} TregP as well as CD25^{-}Foxp3^{hCD2-} CD4SP thymocytes (Tnaïve cells) taken as control. Again, only newly generated CD4SP thymocytes, here identified as CD73^{low} cells as described before (23), were included into the analysis (**Figure S3**). While CD25^{+} TregP displayed a largely methylated Treg cell-specific epigenetic signature comparable to Tnaïve cells, Foxp3^{+} TregP already showed first signs of demethylation particularly in *Eos* and *Helios* (**Figure 2D**).

Together, these data confirm that Foxp3^{+} TregP arise at a more mature developmental stage when compared to CD25^{+} TregP, which is accompanied by a partial demethylation of the Treg cell-specific epigenetic signature genes. Furthermore, the lack of RFP accumulation in the fate-mapping mice strongly suggests that Foxp3^{+} TregP constitute an only transient developmental stage, in agreement with their precursor nature.

CD25^{+} TregP and Foxp3^{+} TregP Show Distinct Responsiveness Toward IL-2

The two-step model of tTreg cell development proposes that after a first TCR-instructive phase CD25^{+} TregP and Foxp3^{+} TregP are subsequently exclusively dependent on signals derived from cytokines, especially IL-2 (14, 15, 18). As we have shown that CD25^{+} TregP and Foxp3^{+} TregP arise at distinct maturation stages, we next wanted to investigate whether the two Treg cell precursor subsets would also show distinct responsiveness toward IL-2. In order to answer this question, we isolated CD25^{+} TregP and Foxp3^{+} TregP as newly generated CD73^{low} CD4SP thymocytes from Foxp3^{hCD2} reporter mice and cultured them in a minimalistic *in vitro* system for 3 or 5 days in presence of IL-2. Based on previously published findings (15) as well as on preliminary own experiments (**Figure S4**), a rather high concentration of IL-2 (50 ng/ml) was chosen, which achieved maximal frequencies of CD25^{+}Foxp3^{+} Treg cells upon culture of the Treg cell precursors. It is important to note that culture of CD25^{+} TregP and Foxp3^{+} TregP in absence of IL-2 does not lead to any induction of Foxp3 and CD25 expression, respectively (15, 18, 23). At the end of the cultures, developing CD25^{+}Foxp3^{hCD2+} Treg cells were FACS-sorted, and further

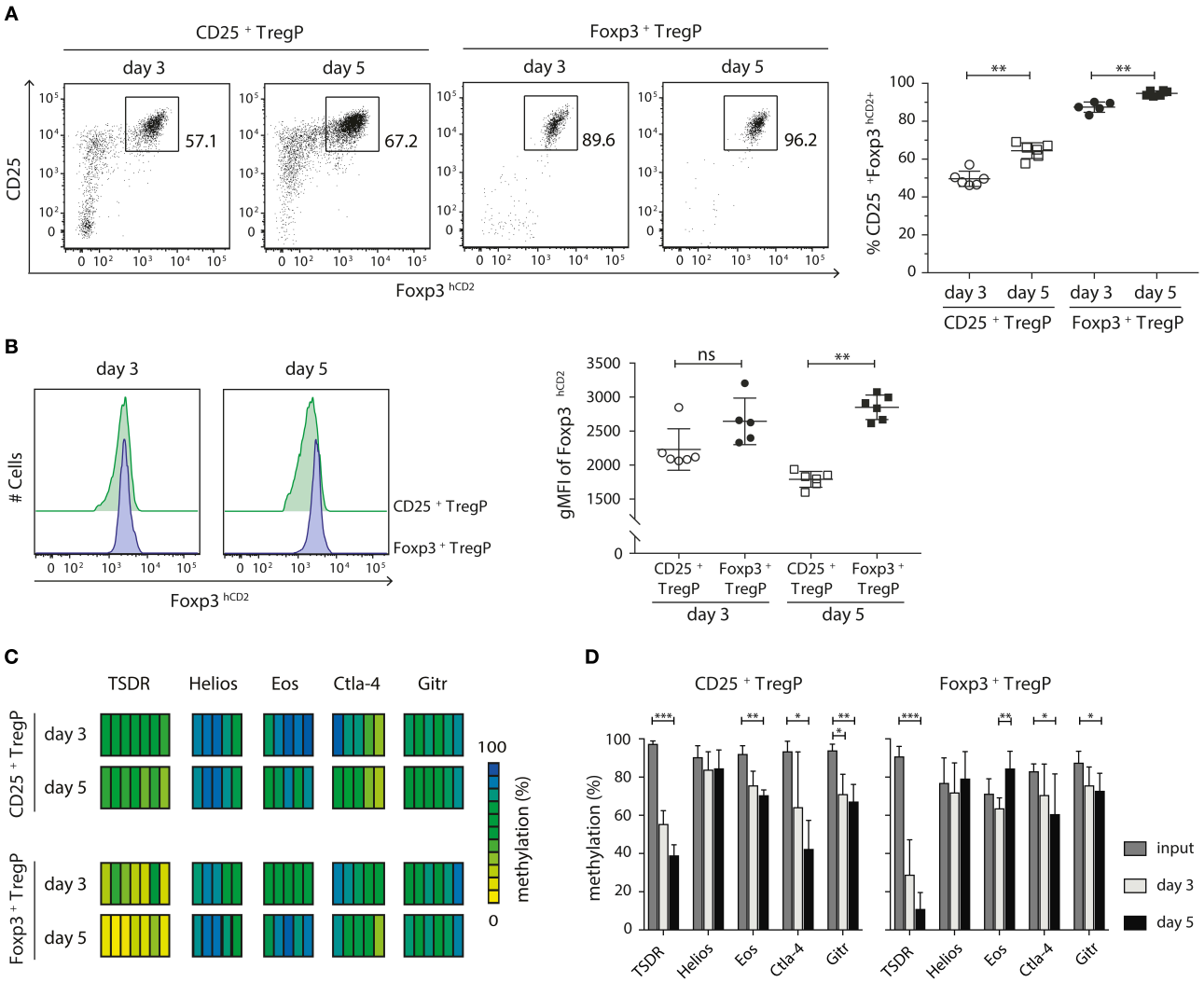

FIGURE 3 | CD25$^+$ TregP and Foxp3$^+$ TregP show distinct responsiveness toward IL-2. Thymocytes were isolated from Foxp3^{hCD2} reporter mice, CD25$^+$ TregP and Foxp3$^+$ TregP pre-gated on newly generated CD73^{low}CD4SP thymocytes were sorted, and sorted Treg cell precursors were cultured in the presence of rmIL-2. At day 3 or 5 of the culture, CD25$^+$Foxp3^{hCD2+} Treg cells were FACS-sorted and analyzed by flow cytometry or prepared for subsequent methylation analysis. **(A)** (Left) Representative dot plots show the gating of CD25$^+$Foxp3^{hCD2+} Treg cells from the indicated cultures. Numbers specify frequencies of cells in indicated gates. (Right) Scatter plot summarizes the data from six independent experiments and bars indicate mean ± SD. Each symbol represents an individual experiment. The significance was calculated using Mann-Whitney test (**$p < 0.01$). **(B)** (Left) Representative histograms depict Foxp3^{hCD2} expression among Treg cells sorted from indicated cultures. (Right) Scatter plots summarize the data from six independent experiments and bars indicate mean ± SD. Each symbol represents an individual experiment. The significance was calculated using Mann-Whitney test (ns = not significant; **$p < 0.01$). **(C,D)** Genomic DNA was isolated from sorted CD25$^+$Foxp3^{hCD2+} Treg cells of the indicated cultures for analysis of the methylation status of TSDR, *Helios*, *Eos, Ctla-4*, and *Gitr*. Cells were pooled from six independent experiments. **(C)** Each bar represents an individual CpG motif. Percentage of methylation is color-coded according to the scale. **(D)** Bar graph summarizes changes in the CpG methylation ratio of the Treg cell-specific epigenetic signature genes in sorted Treg cell precursors (input) and sorted CD25$^+$Foxp3^{hCD2+} Treg cells from the indicated cultures (bars indicate mean ± SD of all CpG motifs). The significance was calculated using Kruskal-Wallis with Dunn's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

flow cytometric analysis as well as pyrosequencing of bisulfite-treated genomic DNA was performed. In line with previously published observations (18, 23), only a fraction (∼50–60%) of CD25$^+$ TregP matured into CD25$^+$Foxp3^{hCD2+} Treg cells upon stimulation with IL-2 (**Figure 3A**). In contrast, the majority (85–95%) of Foxp3$^+$ TregP up-regulated CD25 expression upon stimulation with IL-2 resulting in CD25$^+$Foxp3^{hCD2+} Treg cells (**Figure 3A**), demonstrating a high responsiveness of Foxp3$^+$ TregP to IL-2 signals although they lack expression of the high affinity IL-2 receptor. For both Treg cell precursors, the frequencies of CD25$^+$Foxp3^{hCD2+} Treg cells further increased from day 3 to 5 of culture (**Figure 3A**). Additionally, the expression levels of Foxp3^{hCD2} were elevated in Treg cells arising from Foxp3$^+$ TregP when compared to CD25$^+$ TregP, particularly at day 5 of culture (**Figure 3B**). These data indicate a more pronounced maturation of Foxp3$^+$ TregP in response to IL-2 when compared to CD25$^+$ TregP, which is in line with the observed more mature phenotype of Foxp3$^+$ TregP.

Finally, we assessed the methylation status of the Treg cell-specific epigenetic signature genes in $CD25^+Foxp3^{hCD2+}$ Treg cells generated *in vitro* from the two different Treg cell precursors upon stimulation with IL-2. In any culture, the most pronounced IL-2-induced demethylation was observed at the TSDR (**Figures 3C,D**). Yet, *Ctla-4*, and *Gitr* were also significantly demethylated in both cultured Treg cell precursors. Interestingly, while cultured $CD25^+$ TregP showed a significant demethylation at *Eos*, this epigenetic signature gene was remethylated in $Foxp3^+$ TregP cultured for 5 days (**Figure 3D**). Importantly, the TSDR demethylation was overall more pronounced in cultured $Foxp3^+$ TregP when compared to cultured $CD25^+$ TregP, reaching almost complete demethylation at day 5 (**Figures 3C,D**). Together, these results suggest that IL-2 is mainly acting on the demethylation of the TSDR to ensure stable Foxp3 expression.

DISCUSSION

The majority of $CD25^+Foxp3^+$ Treg cells are known to develop within the thymus, and several recent studies provided insights into the generation and maturation of tTreg cells (13). The two-step model of tTreg cell development proposes that tTreg cells develop from $CD25^-Foxp3^-$ naïve CD4SP thymocytes into $CD25^+Foxp3^+$ Treg cells via a $CD25^+Foxp3^-$ ($CD25^+$ TregP) or a $CD25^-Foxp3^+$ ($Foxp3^+$ TregP) precursor stage, and these two different Treg cell precursors were recently reported to contribute almost equally to the tTreg cell population (23). In this model, the first developmental step is instructed by TCR signaling and co-stimulation, whereas the second step was shown to depend on signals derived from common γ-chain cytokines, particularly IL-2 (14, 15, 18). Importantly, before their egress from the thymus, $CD25^+Foxp3^+$ Treg cells further mature, shown as progressive demethylation of the TSDR along different maturity stages of Treg cells (12). The establishment of a Treg cell-specific hypomethylation pattern, including the TSDR and additional genes such as *Gitr*, *Helios*, *Eos*, and *Ctla-4*, is a prerequisite for the stable suppressive phenotype of Treg cells (9–12). However, specific dynamics of the imprinting of the Treg cell-specific epigenetic signature during tTreg cell development and maturation, and the exact role of IL-2 in directing these processes still remain elusive.

In the present study, we, therefore, performed flow cytometric and methylation analyses on the two Treg cell precursors as well as on Treg cells of different maturation stages. We could demonstrate that $CD25^+Foxp3^+$ Treg cells already display a partially demethylated Treg cell-specific epigenetic signature at the most immature $CD24^{hi}$ stage. These immature Treg cells continuously matured by increasing expression of CD25 and Foxp3 as well as the further progressive establishment of the Treg cell-specific hypomethylation pattern. Importantly, in order to get a precise impression of the events taking place solely within the thymus, we excluded mature Treg cells from our analysis that might have re-circulated from the periphery to the thymus. In addition, we limited our study to "true" Treg cells by analyzing $CD25^+Foxp3^+$ cells rather than the bulk $Foxp3^+$ population. This refined way of analysis explains the small discrepancies between the overall lower TSDR methylation level of $CD24^{hi}$, $CD24^{int}$, and $CD24^{low}$ subsets of tTreg cells obtained in the present study compared to previously published results from our group (12).

We could also confirm that $Foxp3^+$ TregP arise later in ontogeny and are phenotypically at a more mature developmental stage when compared to $CD25^+$ TregP (23). In line with a study from Tai et al., proposing that Foxp3 is lethal for developing thymocytes unless counteracted by cytokine signaling (18), we demonstrated that $Foxp3^+$ TregP represent a very transient population. Interestingly, we could show that $Foxp3^+$ TregP but not $CD25^+$ TregP already display a partial demethylation of the Treg cell-specific epigenetic signature, particularly in *Eos* and *Helios*. These data strongly suggest that at least a part of the Treg cell-specific epigenetic signature is already engraved into the developing tTreg cells before they have entered the very transient $Foxp3^+$ TregP stage, in line with the finding that this unique hypomethylation pattern can be fully established even in the absence of Foxp3 expression (11).

Intriguingly, *in vitro* culture of the two Treg cell precursors in the presence of IL-2 mainly resulted in the progressive demethylation of the TSDR with a more pronounced effect seen for $Foxp3^+$ TregP. The demethylation of the TSDR was reported to be induced by an active process involving enzymes of the Ten-Eleven-Translocation (Tet) family, which act by iterative oxidation of 5mC to 5hmC (12, 24, 32). In this respect, IL-2 was shown to be required for the maintenance of Tet2 at high levels during tTreg cell development. Tet2 further protects the CpG motifs of the TSDR from re-methylation, leading to stable Foxp3 expression in Treg cells (32, 33). These findings support our observation that cultures of Treg cell precursors with IL-2 resulted in the progressive demethylation of the TSDR. This effect was more pronounced in Treg cells arising from $Foxp3^+$ TregP when compared to $CD25^+$ TregP. In line with this, $Foxp3^+$ TregP developed into $CD25^+Foxp3^+$ Treg cells at increased frequencies, and Treg cells arising from $Foxp3^+$ TregP displayed elevated Foxp3 expression levels when compared to Treg cells arising from $CD25^+$ TregP. The superior demethylation observed in Treg cells arising from $Foxp3^+$ TregP may directly result in these increased Foxp3 expression levels, as IL-2 signaling was shown to stabilize Foxp3 expression in Treg cells by activation of STAT5, which binds directly to the demethylated, open TSDR and enhances Foxp3 expression (34–36).

In contrast to the TSDR demethylation, to the best of our knowledge a direct link between IL-2 signaling and the establishment of the Treg cell-specific hypomethylation pattern at the other epigenetic signature genes has not been reported so far. Interestingly, we here could demonstrate that also *Ctla-4* and *Gitr* get significantly demethylated in both Treg cell precursors upon culture with IL-2. Yet, opposing effects were observed for *Eos*, and *Helios* did not show any signs of demethylation. Our observation that, with the exception of the TSDR in cultured $Foxp3^+$ TregP, none of the Treg cell-specific epigenetic signature

genes got completely demethylated upon culture with IL-2 implies the requirement for other factors, lacking in the applied minimalistic *in vitro* culture system, for full acquisition of the Treg cell-specific hypomethylation pattern. In this regard, Owen et al. already has shown a differential need of co-stimulatory signals and other cytokines besides IL-2 for $CD25^+$ TregP and $Foxp3^+$ TregP, in line with their differential localization within the thymus (23).

In conclusion, the results of the present study show that the developmental maturation of tTreg cells is a continuous process, accompanied by the imprinting of the Treg cell-specific epigenetic signature, which endows these tTreg cells with stable Foxp3 expression and stable suppressive properties. The two known tTreg cell precursors display distinct dynamics during the establishment of the Treg cell-specific hypomethylation pattern and further molecular factors involved in this imprinting process need to be elucidated in the future.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

SHe, AT, BP, and TM performed the experiments. YK, NO, SF, SHo, and SS discussed and interpreted the data. SHe, AT, and JH designed the research, interpreted the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02382/full#supplementary-material

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REVIEW
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Foxp3 Post-translational Modifications and Treg Suppressive Activity

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Regulatory T cells (Tregs) are engaged in maintaining immune homeostasis and preventing autoimmunity. Treg cells include thymic Treg cells and peripheral Treg cells, both of which can suppress the immune response via multiple distinct mechanisms. The differentiation, proliferation, suppressive function and survival of Treg cells are affected by distinct energy metabolic programs. Tissue-resident Treg cells hold unique features in comparison with the lymphoid organ Treg cells. Foxp3 transcription factor is a lineage master regulator for Treg cell development and suppressive activity. Accumulating evidence indicates that the activity of Foxp3 protein is modulated by various post-translational modifications (PTMs), including phosphorylation, O-GlcNAcylation, acetylation, ubiquitylation and methylation. These modifications affect multiple aspects of Foxp3 function. In this review, we define features of Treg cells and roles of Foxp3 in Treg biology, and summarize current research in PTMs of Foxp3 protein involved in modulating Treg function. This review also attempts to define Foxp3 dimer modifications relevant to mediating Foxp3 activity and Treg suppression. Understanding Foxp3 protein features and modulation mechanisms may help in the design of rational therapies for immune diseases and cancer.

Keywords: regulatory T cells, Foxp3, post-translational modification, phosphorylation, O-GlcNAcylation, acetylation, ubiquitylation, methylation

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PHENOTYPICAL FEATURES OF REGULATORY T CELLS

Origins, Lineage Definition and Classification, Suppression Mechanism, Epigenetic and Metabolic Features of Treg Cells

$CD4^+CD25^+$ T cells have been identified as a suppressive T cell subset, which have a dominant role in mediating peripheral immune tolerance (1). The Treg population includes two main subsets according to their origins: thymic Treg cells (tTregs) develop with stimulations of self-antigens, cytokines, and costimulatory molecules in thymus; peripheral Treg cells (pTregs) are differentiated from naïve T cells by the act of cytokine TGF-β in periphery. Both tTregs and pTregs require expression of the Foxp3 transcription factor to maintain their lineage stability and effective suppression of immune responses (2, 3).

The definition of a stable Treg lineage can be evaluated by several features, including constitutive expression of Foxp3 and a variety of Treg feature markers (4). Expression of Foxp3 is a prerequisite for Treg lineage commitment and maintenance. Foxp3 expression relies on demethylation of the conserved non-coding sequence 2 (CNS2, also known as the Treg cell-specific demethylated region TSDR) in the *Foxp3* locus. A deletion of CNS2 results in loss of Foxp3 expression during Treg cell expansion and destabilizes Treg cells (5–7). High-resolution quantitative proteomics and transcriptomics approaches have revealed that expression patterns of the core Treg properties, including CD25, CTLA-4, Helios, and *FOXP3* gene TSDR methylation, appear relatively stable in culture *in vitro* (8). The role of Foxp3 in Treg function will be discussed below. Moreover, Treg cells are endowed with unique processes to rapidly respond to environmental cues, and can achieve this through distinct mechanisms of regulation of global or gene-specific mRNA translation. Unlike gene transcription, translational regulation is advantageous for environmental-sensing as it provides a rapid and energetically favorable mechanism to shape the proteome of a given cell, and to tailer cell function to the extracellular context (9). Indeed, distinct translational signatures distinguish Treg and Teff cells (10).

Treg cells are phenotypically diverse in migration, homeostasis, and function (11). Tregs are divided into $CD44^{low}$ $CD62L^{high}$ central Tregs (cTregs) and $CD44^{high}CD62L^{low}$ effector Tregs (eTregs). cTregs are quiescent, IL-2 signaling dependent and long-lived, and they function in the secondary lymphoid tissues to suppress T cell priming; in contrast, eTregs are highly activated and ICOS signaling dependent with potent suppressive function in specific non-lymphoid tissues to dampen immune responses (12). eTregs have increased mTORC1 signaling and glycolysis compared with cTregs. Consistently, inhibition of mTORC1 activity by administration of rapamycin (mTORC1 inhibitor) promotes generation of long-lived cTreg cells *in vivo* (13). Treg cells lacking Ndfip1, a coactivator of Nedd4-family E3 ubiquitin ligases, elevate mTORC1 signaling and glycolysis, which increases eTreg cells but impairs Treg stability in terms of Foxp3 expression and pro-inflammatory cytokine production (14).

Treg cells suppress immune response via multiple mechanisms [as reviewed in (15–17)]. Treg cells highly express CD25 (the IL-2 receptor α-chain, IL-2Rα) and may compete with effector T cells leading to consumption of cytokine IL-2 (18). Treatment with low-dose rhIL-2 selectively promotes Treg frequency and function, and ameliorates diseases in patients with systemic lupus erythematosus (SLE) (19). The constitutive expression of CD25, a direct target of Foxp3, is essential to engage a strong STAT5 signal for Treg proliferation, survival, and Foxp3 expression (20). CTLA-4 activation can down-regulate CD80 and CD86 expression on antigen-presenting cells (21). Treg cells also produce inhibitory cytokines, IL-10, TGF-β, and IL-35, to enhance immune tolerance along with cell-contact suppression (22–24). Treg cells may mediate specific suppression by depleting cognate peptide-MHC class II from dendritic cells *in vivo* (25). Of note, Treg cells recognize cognate antigen and require T cell receptor (TCR) signaling for optimal activation, differentiation, and function (26). Polyclonal expanded Treg cell mixed populations exhibit suppressive potency for certain autoimmune diseases (27). Engineering Treg cells with antigen-specific TCR appears to lead to antigen-specific suppression with increased potency (28).

Treg cells exploit distinct energy metabolism programs for their differentiation, proliferation, suppressive function, and survival (29, 30). Rather than glucose metabolism, Treg cells have activated AMP-activated protein kinase (AMPK) and use lipid oxidation as an energy source. AMPK stimulation by Met can decrease Glut1 and increase Treg generation (31). Further proteomic analysis showed that fresh-isolated human Treg cells are highly glycolytic, while non-proliferating Tconv cells mainly use fatty-acid oxidation (FAO) as an energy source. When cultured *in vitro*, Treg proliferation and suppression require both glycolysis and FAO, while Tconv cells mainly rely on glucose metabolism for proliferation and function. This finding indicates that Treg cells and Tconv cells may adopt different metabolic programs *in vitro* and *in vivo* (32). Treg cells cannot only use anabolic glycolysis to produce sufficient fundamental building blocks to fuel cell expansion, but also efficiently generate ATP energy via catabolic fatty acid oxidation (FAO) driven oxidative phosphorylation (OXPHOS) by the mitochondria to support activation and suppression function (33).

Treg cells have greater mitochondrial mass and higher ROS production than Tconv cells. Tregs are more vulnerable to OXPHOS inhibition, which underscores the unique metabolic features of Treg cell (34). Loss of subunit of the mitochondrial complex III RISP in Treg cells diminishes oxygen consumption rate (OCR), but increases in glycolytic flux. Treg cells require the activity of mitochondrial complex III to maintain Treg feature gene expression and suppression (35). Foxp3 expression increases mitochondrial respiratory capacity and promotes ATP generation through oxidative phosphorylation. Increased fatty acid metabolism can protect Treg cells from fatty acid–induced apoptosis accordingly (36). Toll-like receptor (TLR) signals activate PI(3)K-Akt-mTORC1 signaling and glycolysis to promote cell proliferation, however, Foxp3 opposes PI(3)K-Akt-mTORC1 signaling but increases oxidative phosphorylation for effector function. Thus, Foxp3 and inflammatory TLR signals control the proliferation and suppressive function of Treg cells through balancing the metabolic activity between glycolysis and oxidative phosphorylation (37). Genetic deletion of mTOR gene (*Frap1*) or pharmacological inhibition of mTOR activity by rapamycin promotes Treg cell maintenance (38, 39). A recent study shows that the deficiency of Foxp3 dysregulates mTORC2 signaling, which promotes aerobic glycolysis and oxidative phosphorylation. Genetic deletion of the mTORC2 adaptor gene *Rictor* or pharmacological treatment with mTOR inhibitors antagonizes the Teff cell-like program and restores the suppressive function of *Foxp3*-deficient Treg cells (40).

Features of Tissue-Resident Treg Cells

Treg cells adopt distinct patterns of tissue- or immune-context-specific suppression mechanisms (41). Treg function *in vivo* requires a timely recruitment and/or accumulation in non-lymphoid tissues where immune responses are frequently

occurring. In these sites, Treg cells adapt their function (i.e., effector mechanism) based on local immune mediators (42). For instance, TNFα-induced dephosphorylation of FOXP3 at Ser-418 by protein phosphatase 1 (PP1) compromises Treg suppressive activity within the inflamed synovium, which is responsible for the pathogenicity of rheumatoid arthritis (43). Here, we attempt to review some unique features and underlying mechanisms of tissue-resident Treg cells in skin, visceral adipose tissue and tumor, which has attracted extensive research interests recently (44–46).

Skin is an immunologically active organ that protects against pathogen invasion but prevents collateral tissue damage (47). Both murine and human skin contain a large number of Tregs, which accumulate through multiple chemokines and ligands, such as CCR4, CCR6, CCL17, CCL20, and CCL22 (48, 49). Functionally, Tregs inhibit proinflammatory cytokine IFN-γ production and macrophage accumulation in wounded skin to facilitate cutaneous wound closure and healing (50). Skin-resident Treg cells localize to hair follicles and facilitate stem cell-mediated hair follicle regeneration through Notch-Jagged signaling pathway (51). Tregs contribute to the establishment of immune tolerance to commensal microbes in neonatal skin and colon (51–53).

Visceral adipose tissue (VAT)-resident Treg cells guard against abnormal inflammation of the adipose tissue in obesity, type 2 diabetes and atherosclerosis (54, 55). Progressively decreased frequency of Tregs in visceral adipose tissue leads to the adipose inflammation and insulin resistance in obese animal models, while the induction of Tregs by administration of IL-2 contained complex or anti-CD3 monoclonal antibody can ameliorate glucose tolerance and insulin sensitivity (56, 57). VAT-Treg cells show unique gene signatures implicated in leukocyte migration, extravasation, and cytokine production (56). Peroxisome-proliferator-activated receptor γ (PPARγ) acts as a crucial orchestrator for the accumulation, phenotype, and function of VAT-Treg cells; a deficiency of PPARγ in Treg cells down-regulates the specific VAT Treg gene expression signature and Treg frequency in the visceral adipose tissue particularly (58). VAT-Treg cells maintain adipose tissue homeostasis by catabolizing prostaglandin E2 (PGE2) into the metabolite 15-keto PGE2, which limits conventional T cell activation and proliferation in visceral adipose tissue (59).

Treg cells are often enriched in tumor tissue, and a high ratio of tumor-infiltrating Treg cells (TIL-Tregs) to effector T cells generally predicts poor clinical outcomes of certain types of cancers, including ovarian cancer, breast cancer, melanoma, and hepatocellular carcinoma (60). Depleting intra-tumoral Tregs by treatment with immune checkpoint inhibitor monoclonal antibody against cytotoxic T lymphocyte–associated antigen 4 (anti-CTLA-4) promotes anti-tumor immune activity (61, 62). A recent study shows that the antitumor efficacy of anti-CTLA-4 can be enhanced by the combination therapy with Toll-like receptor 1/2 (TLR1/2) ligand via a FcγRIV-dependent manner (63). The origins of TIL-Tregs are diverse, including: (1) selective recruitment to tumor sites; (2) conversion from conventional $CD4^+$ T cells; (3) Treg cell expansion (64). TIL-Tregs exhibit Foxp3 TSDR hypomethylation, and are highly proliferated and apoptotic due to the oxidative stress within tumor lesions (65, 66). Treg cells can thrive in tissues with ischemic injury or the tumor microenvironment, characterized by low-glucose and high-lactate. Treg cells favor oxidation of L-lactate to pyruvate to increase the intracellular NAD:NADH ratio, and resist the suppressive effects of L-lactate on cell proliferation (67). In addition, Tregs are endowed with the relative advantage of a circuitry of glycolysis, fatty acid synthesis, and oxidation to prevail over conventional T cells in the hostile tumor microenvironment (68).

Overall, tissue-resident Treg cells play unique roles in distinct non-lymphoid tissues via multiple molecular mechanisms, including regulation by post-translational modification of FOXP3 protein (which will be discussed in detail in the section of "Post-translational modifications of Foxp3"). It holds promise to harness the behavior and function of tissue-resident Tregs, specifically to treat certain immune disorders, such as skin autoimmunity and regeneration, obesity, type 2 diabetes and cancer.

FOXP3 IS A DOMINANT REGULATOR OF TREG CELLS

The Forkhead box (FOX) protein superfamily of transcriptional regulators play pleiotropic roles in cell proliferation, differentiation, survival, and apoptosis during embryonic development and homeostasis of adult tissues (69). The FOXP family include FOXP1, FOXP2, FOXP3, and FOXP4 members. Foxp1 interacts with and regulates Foxp3 chromatin binding to coordinate Treg cell suppressive function and homeostasis (70–72). In humans, the FOXP3 gene (originally named JM2 or Scurfin) is found on the X-chromosome at Xp11.23-Xq13.3 (73). Genetic analysis has demonstrated that Treg cell dysfunction caused by the FOXP3 mutation is responsible for the immune dysregulation polyendocrinopathy enteropathy X-linked syndrome [IPEX, also called X-linked autoimmunity–allergic dysregulation syndrome (XLAAD)], a recessive immune disorder occurring in newborns and children (74). In mice, conditional deletion of Foxp3 in $CD4^+$ T cells leads to fatally lymphoproliferative autoimmunity, which includes high IgE levels, enteropathy, type 1 diabetes and failure to thrive, while ectopic expression of Foxp3 can re-program conventional $CD4^+$ T cells as anti-inflammatory Treg cells (75).

The Foxp3 protein contains multiple structural domains. There is a proline-rich N-terminal domain that interacts with many molecular factors to regulate transcriptional activity. Another central zinc-finger and leucine-zipper domain is involved in oligomer formation, and a conserved C-terminal forkhead/winged helix domain (FKH) is responsible for DNA binding (76). Chromatin immuno-precipitation combining genome-wide analysis revealed that Foxp3 interacts with up to ∼700 genes. Interestingly, Foxp3 can activate or repress its target genes to cooperatively regulate Treg cell development, function, and homeostasis (77, 78). Foxp3 protein complexes define the transcriptional network of Treg cells (79). Foxp3 forms heterogeneous super-complexes of 400–800 kDa and associates

with 361 partner proteins of which ∼30% are transcription related. These proteins include Foxp1, Foxp4, Stat3, IKZF1, Runx1, and GATA-3 (71, 72). These interactions highlight the master role of Foxp3 in programming Treg cell lineage.

FOXP3 mutations that occur in IPEX patients include missense point mutations, frameshift, and missplicing, causing a premature stop codon, as well as mutations in the polyadenylation site. The missense mutations can preserve a normal or reduced expression of FOXP3 in IPEX patients. Although various distinct FOXP3 mutations have been reported with IPEX patients, individuals with the same mutation can develop disease manifestations that are not similar, indicating heterogeneity of severity among IPEX cases (80). IPEX patients may present with different intestinal lesions (81), and similar genotypes come out with different symptoms and severity (82); which suggests the complex relevance of genotype and phenotype in IPEX patients, and reflects complex intracellular interactions and post-translational modifications of FOXP3 (83).

POST-TRANSLATIONAL MODIFICATIONS OF FOXP3

Post-translational modifications (PTMs) of proteins link cellular signals to the functional properties. The transcriptional activity of Foxp3 can be modulated by various post-translational modifications, such as phosphorylation, O-GlcNAcylation, acetylation, ubiquitination, and methylation (**Figure 1**).

Phosphorylation and Dephosphorylation of Foxp3

Protein phosphorylation, occurring at serine, threonine or tyrosine resides of proteins, is a reversible and transient modification catalyzed by certain kinases and phosphatases. Protein phosphorylation is involved in protein intracellular stability, interaction, and localization. TCR engagement triggers a signaling cascade with rapid phosphorylation events, and reprograms the proteomes and bioenergetic features for T cell activation, proliferation, and differentiation (84). In humans, TCR stimulation can induce transient FOXP3 expression (85). TCR signaling enhances Foxp3 phosphorylation through incubation of anti-CD3/CD28 antibodies or pharmacological treatment with PMA (phorbol 12-myristate 13-acetate) and Ionomycin (86). A recent report found that TCR stimulation can activate TAK1-NLK signaling, leading to phosphorylation of Foxp3, which decreases its interaction with the STUB1 E3-ubiquitin ligase to modify ubiquitination and proteasome-mediated degradation rates. Using mass spectrometry, seven distinct phosphorylated residues (S19, S156, S189, S273, S278, S295, and T341) of Foxp3 have been identified upon the co-expression of NLK in cell line. Although there may be other substrates targeted by NLK kinase other than Foxp3, Treg cell-specific NLK deficiency results in auto-inflammation and renders animals susceptible to induced experimental autoimmune encephalomyelitis (EAE) (87).

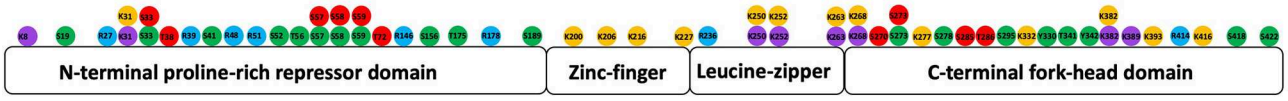
Levels of the inhibitor of the cyclin-dependent kinases (CDKs) p27kip1 are up-regulated in anergic T cells. CDK2 is a direct target of p27kip1 and CDK2 promotes cytokine production by $CD4^+$ T cells, while limiting Treg function. Treg cells lacking CDK2 had increased capacity to inhibit Tconv cell proliferation and ameliorate immunopathogenesis in colitis (88). The N-terminal domain of Foxp3 protein contains several putative CDK motifs. CDK2 kinase phosphorylates Ser-19 and Thr-175 residues of Foxp3. Mutation of CDK motifs via a substitution of Ser/Thr to Ala increases Foxp3 protein stability and transcriptional activity, which manifests that Foxp3 phosphorylation by CDK2 kinase negatively regulates Treg function (89). These findings demonstrate that CDK2 negatively regulates Treg function and peripheral tolerance by phosphorylating Foxp3.

The Foxp3 N-terminal domain is important for Foxp3 transcriptional repression and nuclear transport (90, 91). Pim-2 kinase, an oncogenic serine/threonine kinase, can phosphorylate multiple sites of Foxp3 N-terminal domain as identified by mass spectrum analyses. The deficiency of Pim-2 increases Treg suppression in *in vitro* assay, and *Pim-2*-deficient mice appear resistant to DSS-induced acute colitis (92). Pim-2 kinase can promote rapamycin-resistant survival, growth and proliferation of lymphocytes, including Treg cells (93, 94). The Pim-1 kinase phosphorylates Ser422 of the forkhead domain of human FOXP3, which attenuates FOXP3 DNA binding activity and down-regulates expression of Treg feature genes. Knockdown of Pim-1 in Tregs enhances suppressive activity (95). These findings demonstrate that phosphorylation of Foxp3 by Pim-1 and Pim-2 negatively regulates Foxp3 transcriptional activity and Treg suppressive function. Kaempferol is a natural flavonoid found in vegetables and fruits, which may reduce PIM1-mediated FOXP3 phosphorylation at S422 and enhance Treg cell suppressive capability. The anti-inflammatory effect of *Kaempferol* may facilitate therapies of certain autoimmune diseases (96).

Phosphorylation of the Ser-418 located in the FOXP3 C-terminal forkhead domain plays a positive role in regulating Treg suppressive function (43). TNF-α induces protein phosphatase 1 (PP1), which can dephosphorylate Ser-418 of FOXP3 and limit Treg cell activity, while increasing pathogenic Th17 and Th1 CD4 T cells within the inflamed synovium. TNF-α antagonist therapy (treatment with TNF-α-specific antibody: Infliximab) increased Treg cell function in patients with rheumatoid arthritis (43). Ser-418 phosphorylation of FOXP3 affected C-terminal cleavage of Foxp3 protein by proprotein convertase (PC) (97) and also modulated Foxp3 DNA affinity by interactions with other proximal modification, such as acetylation (98). FOXP3 can be phosphorylated at the Tyr-342 site by Lymphocyte-specific protein tyrosine kinase (LCK) in the MCF-7 cell line. Tyr-342 phosphorylation of FOXP3 down-regulates expression of *SKP2*, *VEGF-A*, and *MMP9* genes (99). Taken together, Foxp3 undergoes phosphorylation modifications, and these modifications can either positively or negatively modulate Treg function according to the various disease settings.

O-GlcNAcylation Modification of Foxp3

Protein O-GlcNAcylation modification occurs at serine and threonine residues, as well as phosphorylation, which is catalyzed by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) oppositely. O-GlcNAcylation may counteract ubiquitination



Type of PTMs	Modified sites of Foxp3	Modifier	Roles in Treg suppression	References
Phosphorylation	S418	PP1	Positive	Nie, *et al.* 2013
	Y330, Y342	LCK	Unknown	Nakahira, *et al.* 2013
	S19, T175 (mouse)	CDK2	Negative	Morawski, *et al.* 2013
	S422	PIM1	Negative	Li, *et al.* 2014
	S33, S41, S52, T56, S57, S58, or S59 (mouse)	PIM2	Negative	Deng, *et al.* 2015
	S19, S156, S189, S273, S278, S295, T341	NLK	Positive	Fleskens, *et al.* 2019
O-GlcNAcylation	S33, T38, S57, S58, S59, T72, S270, S273, S285,T286 (mouse)	OGT/OGA	Positive	Liu *et al.* 2019
Acetylation	K31, K263, K268	p300/SIRT1	Positive	Kwon *et al.* 2012
	K250, K252	p300	Negative	Song *et al.* 2012
	K8, K382, K389	Unknown	Positive	Tao *et al.* 2012; Samanta *et al.* 2012
Ubiquitylation	K227, K250, K263, K268	Stub1	Negative	Chen *et al.* 2013
	K249, K251, K262, K267, K393 (mouse)	USP7 deubiquitinase	Positive	Loosdregt *et al.* 2013
	K206, K216, K227, K252, K277, K332, K393	USP21 deubiquitinase	Positive	Li *et al.* 2016
	K31, K200, K250, K263, K268, K382, K393, K416	RNF31	Positive	Zhu *et al.* 2018
	K262 (mouse)	TRAF6	Positive	Ni *et al.* 2019
Methylation	R51	Unknown	Unknown	Geoghegan *et al.* 2015
	R48, R51	PRMT1	Positive	Kagoya, *et al.* 2019
	R27, R51, R39, R146, R178, R236, R414	PRMT5	Positive	Nagai, *et al.* 2019

FIGURE 1 | Post-translational modifications of Foxp3 and their roles in Treg suppression. A schematic representation shows the Foxp3 protein structure domains and post-translational modification sites **(top)**. The table lists the kind of modification, modified sites, modifier enzymes, and roles in Treg suppression **(bottom)** (Guoping Deng and Mark I. Greene).

to stabilize FOXP3 protein, and loss of O-GlcNAcylation destabilizes FOXP3 protein. Deficiency of OGT in Treg cells leads to lethal autoimmune diseases in mice (100).

Acetylation of Foxp3 by Histone Acetyltransferases

Acetylation occurs on histone proteins and other cellular proteins (101). Protein acetylation is catalyzed by histone acetyltransferases (HTAs) and histone/protein deacetylases (HDACs) oppositely. HATs can transfer the acetyl moiety of acetyl-coenzyme A to N^{α}-amino groups of methionine residue or the N^{ε}-amino groups of lysine residues on proteins. N^{α}-acetylation occurs as a co-translational process of protein N-terminal methionine cleavage (102), and is a reversible process that modulates protein biological activity in response to internal or external cell stimuli (103).

Based on structural and functional similarity of their catalytic domains, HATs can be grouped into three main families: (1) the Gcn5/PCAF family including Gcn5, PCAF, and related proteins; (2) the p300/CBP family; (3) the MYST family are involved in control of transcription and cell growth and survival (104).

TIP60, p300, and CBP, orchestrate multiple aspects of Treg development, function, and lineage stability (105). TIP60 belongs to the MYST HAT family. The TIP60 histone acetylase complex also plays an important role in DNA repair and apoptosis (106). TIP60 can act as both a transcriptional co-repressor (107, 108) and a co-activator (109). TIP60 is the first identified HAT that acts as an essential subunit of the FOXP3 repression complex. The N-terminal 106–190 aa of FOXP3 associates with TIP60 and HDAC7. The FOXP3–TIP60–HDAC7 complex is required for IL-2 repression by Foxp3 in T cells (110).

p300 acetylates Foxp3 to prevent proteasome-mediated degradation and increase Foxp3 protein levels, as well as Foxp3-mediated transcriptional repression of IL-2 production (111). Mass spectrometry analysis has identified three acetylation sites in murine Foxp3 (K31, K262, and K267), upon co-expression with p300 acetyltransferase in 293T cells (112). A conditional deletion of *Ep300* (which encodes p300) in Treg cells compromises Treg suppressive capability and leads to autoimmunity at around 10 weeks of age. On the other hand, deficiency of p300 impairs Treg cell infiltration into tumor, accompanied with enhanced anti-tumor immunity (113). Administration of the p300 inhibitor C646 or a peptidic p300i (Lys-CoA-Tat) abrogates Treg cell–dependent allograft survival, while increasing the anti-tumor immune responses in animal tumor models (113, 114).

CBP, a p300 paralog, is also important in regulating Treg function in certain inflammatory or lymphopenic conditions. Double-deletion of *CBP* and *p300* in Treg cells leads to fatal autoimmunity by 3–4 weeks of age (115). Likewise, TIP60 and p300 cooperatively regulate FOXP3 activity. p300 interacts with TIP60 and facilitates autoacetylation of TIP60 at K327. This modification increases TIP60 protein stability and promotes FOXP3 acetylation. Reciprocally, TIP60 promotes p300 acetylation and HAT activity as well (116). In contrast to the modest phenotype of *p300*-deficient mice, a deficiency of TIP60

in Tregs results in severe and fatal autoimmune diseases at an early age. These studies indicate that TIP60 plays a more unique and dominant role than p300 (for which there are similar and redundant HAT), in terms of regulation of Treg cell development and function (116). A recent study by Bin Dhuban et al. elegantly shows that some inheritable mutations in the forkhead domain of FOXP3 can specifically disrupt FOXP3-TIP60 association, in turn, compromising human Treg cell development and function. Restoring FOXP3-TIP60 in this setting with allosteric modification of TIP60 also rescued Treg cell function (117, 118).

FOXP3 forms dynamic homo- or hetero- dimer via its zinc-finger and leucine-zipper domain. This dimer structure is characterized as a two-stranded anti-parallel a-helical coiled-coil. The crystal structure indicates that lysine residues, K250 and K252 of FOXP3, are electrostatically involved in the interface network for Foxp3 coiled-coil dimerization. Acetylation of K250 and K252 of FOXP3 by p300 results in dimer relaxation and down-regulates Foxp3 suppressive activity (119). Of note, FOXP3 acetylation and related chromatin DNA binding activity were induced by treatment of TGF-β, through residues other than K250 and K252 (86, 119). These studies suggest a work model that regulates the Treg suppression by integrating the conformation, dimerization and post-translational modification of the FOXP3 protein (**Figure 2**).

Deacetylation of Foxp3 by Histone Deacetylation Enzymes

Eighteen human histone deacetylation enzymes (HDACs) have been identified and grouped into four subsets based on their molecular phylogenetic analysis of primary structure, intracellular localization, and homology to yeast counterpart enzymes (120). Mechanistically, the classical (including classes I, II, and IV) HDAC enzymes depend on zinc ions (Zn^{2+}), whereas the Sirtuins (class III) HDACs utilize nicotinamide adenine dinucleotide (NAD^{+}) as a coenzyme (121).

Treg cells express 11 classical Zn^{2+}-dependent HDACs, and several NAD-dependent HDACs belonging to the Sirtuin family. Although targeting HDAC enzymes in mediating multiple aspects of Treg cell function has received extensive attention (122–124), we focus on reviewing the direct effects of HDAC on Foxp3 protein acetylation and related Treg function regulation. Treatment with a pan-HDACi, trichostatin-A (TSA) boosts the production of thymic $Foxp3^{+}$ Treg cells, Treg feature gene expression, Treg suppression and Foxp3 protein acetylation. Combined therapy with TSA and rapamycin (TSA-RPM) can extend cardiac or islet allograft survival in animal models (75). Moreover, HDAC inhibitors, including sodium butyrate, valproate acid, SAHA, MS-275, Bufexamac, and BML210, can increase human Treg suppression (125, 126).

HDAC inhibitor therapy has been examined for its ability to modify Treg function *in vivo* and effects on protection of allograft and prevention of autoimmunity (127). The class III HDAC SIRT1 also decreases Foxp3 acetylation and Treg stability. Pharmacological treatment with either the HDACs pan-inhibitor, TSA, or SIRT specific inhibitor, NAM, increases Foxp3 levels and Treg suppressor capacity in an *in vitro* suppression assay. Treatment with the SIRT activator, resveratrol, decreases numbers of $FOXP3^{+}$ cells in human PBMC and skin samples (111). Deficiency of Sirt1 promotes Foxp3 stability in iTreg cells and restrains the generation of pathogenic T cells. Administration of the Sirt-1 inhibitor Ex-527 attenuates GVHD but preserves the therapeutic effect of graft-vs.-leukemia (128).

HDAC6, HDAC9, and Sirt1 may deacetylate Foxp3, however, these HDACs regulate Foxp3 gene expression by affecting different transcription factors. HDAC6, HDAC9, and Sirt1 have shared and individual mechanisms of action, and the combined loss of HDAC6, HDAC9, and Sirt1 activity may augment Treg function (129–131).

Ubiquitylation of Foxp3

Protein ubiquitylation modification is catalyzed by a sequential and concerted action of three distinct classes of enzymes: the ubiquitin-activating enzyme, E1, the ubiquitin-conjugating enzyme, E2, and the ubiquitin-ligase, E3 (132). Ubiquitin consists of a 76-amino acid polypeptide that has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), each of which may participate in the formation of diverse poly-ubiquitin chains. K48-linked polyubiquitin chains represent targeting signals for proteasomal degradation, while K63 polyubiquitylation plays non-proteolytic roles, including DNA damage response, receptor endocytosis and protein trafficking (133).

Ubiquitylation signals regulate multiple aspects of immune cell function and immune response (134). Upon T cell activation, the non-degradative ubiquitylation, including K29, K33, and K63 polyubiquitylation, increases largely in $CD4^{+}$ T cells, which suggests the importance of non-degradative ubiquitylation in T cell signaling (135).

As the master regulator of the Treg cell lineage, Foxp3 protein level and turnover rate determine Treg cell identity and function. Inflammatory stress destabilizes Treg cell functions (136, 137). The Foxp3 protein undergoes polyubiquitylation and proteasome-based degradation especially under certain stressful conditions. HIF-1α promotes expression of Th17 signature genes and Th17 cell development, but attenuates Treg development by enhancing Foxp3 ubiquitylation and proteasomal degradation (138). Stimulation by proinflammatory cytokines and lipopolysaccharides (LPS) promotes Foxp3 K48-linked polyubiquitination by the E3 ubiquitin ligase, Stub1, in an Hsp70-dependent manner. The absence of endogenous activity of Stub1 or Hsp70 prevents Foxp3 from proteasomal degradation (139).

Upon TCR stimulation, E3 ubiquitin ligase, Cbl-b, together with Stub1, targets Foxp3 for ubiquitylation and degradation; and a deficiency of Cbl-b can partially rescue defective development of thymic Treg cells in $Cd28^{-/-}$ mice (140). Cimetidine (CIM) can promote immune responses, suppress Treg cell function via activation of PI3K-AKT-mTOR signaling and increase of Stub1-mediated degradation of Foxp3, respectively (141). As mentioned above, Foxp3 phosphorylation by NLK prevents its association with the STUB1 and increases Foxp3 protein stabilization (87). These studies highlight the critical role of Stub1

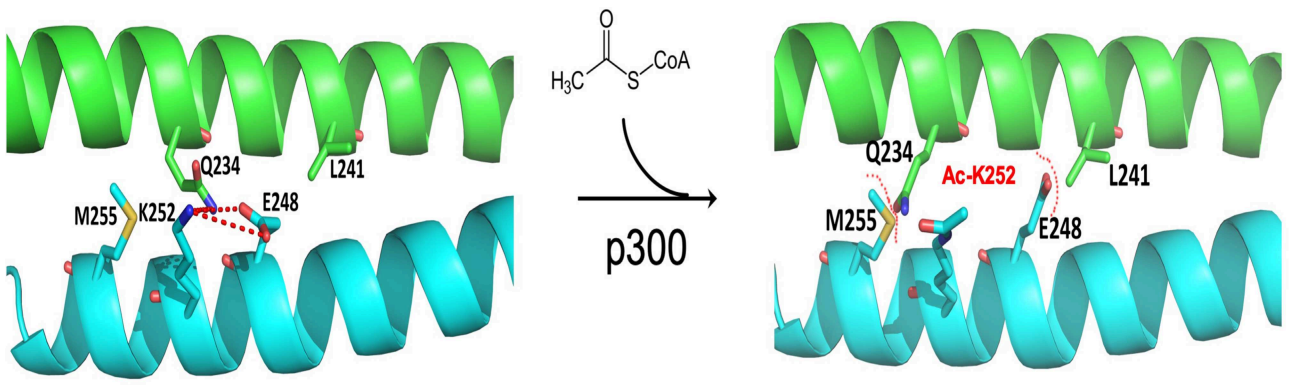


FIGURE 2 | Structure model of acetylation at FOXP3 K252 downregulating suppression by destabilizing homodimerization. FOXP3 forms dynamic homodimer via zinc-finger and leucine-zipper domains, which featured as a two-stranded, anti-parallel a-helical coiled-coil. The inter-subunit hydrogen bond formed between Q234 and E248 stabilizes FOXP3 dimer via electrostatic interactions. The Q234-E248 hydrogen bond depends on the unique conformation of the E248 side chain held by K252 residue **(left)**. Acetylation of K252 by p300 neutralizes the positive charges of K252 side chain and decreases its interaction with E248, and as a consequence, breaks the Q234-E248 inter-subunit hydrogen bond. E248-L241 and Q234-M255 form the steric tension to relax and destabilize the FOXP3 homodimerization **(right)**. (Xiaomin Song, Guoping Deng, and Mark I. Greene).

ubiquitin E3 ligase in mediating Foxp3 protein level and Treg suppressive function.

Besides K48-linked ubiquitylation, Foxp3 also undergoes non-proteolytic ubiquitylation. Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) mediates K63-linked polyubiquitylation (142). TRAF6 plays an essential role in maintaining Treg cell function to inhibit Th2 type autoimmunity (143). A recent study found that deficiency of TRAF6 in Treg cells compromises Treg suppression *in vivo*. Mechanistically, TRAF6 interacts with FOXP3 and mediates K63 polyubiquitination at lysine 262 residue, which ensures proper nuclear localization of FOXP3 and facilitates FOXP3 transcriptional activity in Tregs. Deficiency of TRAF6 in Tregs enhances anti-tumor immunity (144). The E3 ubiquitin ligase ring finger protein 31 (RNF31) catalyzes FOXP3 atypical ubiquitylation, and promotes FOXP3 protein stability and Treg suppression. RNF31 expression has been correlated with intratumoral Treg cell activities in gastric cancer, indicating a potential role of RNF31 in tumor immunity (145).

Deubiquitylation of Foxp3

Protein ubiquitylation can be reversed by the deubiquitinases (DUBs, also known as deubiquitinating enzymes) (146). The deubiquitinases consist of five families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins and JAB1/ MPN/MOV34 metalloenzymes (JAMMs). The UCH, USP, OTU, and Josephin families are Cys proteases, whereas the JAMM/MPN+ family members are zinc metalloproteases (147).

It has been well-documented that deubiquitinases are involved in T cell development, activation, differentiation and tolerance (148). The K63-specific deubiquitinase Cylindromatosis (CYLD) negatively regulates CARMA1, which is required for NF-kB activation and IL-2 receptor signaling. A deficiency of CYLD causes constitutive NF-kB activation and enhanced TGF-b signaling, which increases the frequency of Treg cells in peripheral lymphoid organs and promotes Treg cell differentiation *in vitro* (149, 150). Similarly, Treg cell number increases in a non-functional CYLD splice variant $CYLD^{ex7/8}$, however, the suppressive capacity is impaired and correlated with decreased expression of CD25 and CTLA4 (151).

USP7 is upregulated in Treg cells, and the ectopic expression of USP7 decreases Foxp3 polyubiquitylation and increases Foxp3 expression. Treg cells pretreated with deubiquitinase inhibitor enhance their modifying functions in adoptive transfer induced colitis (152). In addition, USP7 promotes Treg suppression by enhancing the multimerization of Tip60 and Foxp3 (153). USP21 associates with GATA3 and Foxp3 transcriptional factors. Mice depleted of Usp21 in Treg cells develop Th1-type inflammation (154, 155). USP4 stabilizes the IRF8 protein via a K48-linked deubiquitinase, which promotes the suppressive function of Treg cells (156).

Methylation of Foxp3

Arginine methylations are mainly catalyzed by the Protein Arginine Methyltranasferase (PRMT) family. PRMT family can be divided into 3 subfamilies: the Type 1 PRMTs include PRMT1, 2, 3, 4 (also known as CARM1), and 6, which asymmetrically methylate target arginine residues; the Type 2 PRMTs include PRMT5 and 9, which symmetrically methylate target arginine residues; the Type 3 PRMT7 mono-methylates target arginine residues (157, 158).

T cell activation induces protein methylation modification (159). Employing isomethionine methyl-SILAC (iMethyl)-SILAC and mass spectrometry approaches, various transcription factors, including Foxp3, that influence T cell differentiation and lineage specificities, have been identified as being methylated (160). FOXP3 can be di-methylated at arginine(R) 51 position, although whether this methylation is asymmetry or symmetry, and which PRMT is responsible for this modification both remain unclear (129). More recent mass spectrometric analysis

reveals that human FOXP3 contains several possible di-methylation sites at R27, R51, and R147 residues (161). PRMT1 and PRMT5 are reported to be involved in FOXP3 methylation (162). PRMT5 preferentially binds to FOXP3, and a conditional deletion of PRMT5 gene in Tregs leads to deadly scurfy-like autoimmune diseases. Lymph node Tregs from those animals showed significantly less suppressive function, indicating that PRMT5 is essential for Treg function. Site-mutation of R51K dramatically decreases symmetric arginine signals, as detected by symmetric arginine specific antibody sym10, suggesting that PRMT5 di-methylates FOXP3 at R51. Of note, the arginine methylation at the N-terminal position of FOXP3 regulates Treg function via a Foxp3-DNA-binding-activity independent manner. Pharmacological ablation of PRMT5 activity by DS-437 can enhance the anti-tumor efficacy of anti-erbB2/neu monoclonal antibody target therapy by reducing human Treg functions (161).

Targeting protein methylation modification provides novel insights into clinical therapeutic designing for certain diseases, including autoimmune diseases and cancer. PRMT5 forms a complex with MEP50, an essential cofactor, which then causes higher enzymatic activity and allows the binding to target proteins. PRMT5 uses S-adenosyl methionine (SAM) for transferring a methyl group to the target substrate. It is also known that PRMT5 forms a large complex with different cofactors, for changing its target proteins (158). PRMT5 is highly expressed in a variety of cancers, and several inhibitors have been developed that target PRMT5 for cancer therapy (157). PRMT5 forms a large complex and has several different cofactors; the effect of PRMT5 inhibitors may be dependent on the screening strategy, such as substrate- or SAM- competitive. SAM-competitive inhibitor, DS-437, has more efficient inhibition of FOXP3 methylation than the substrate-competitive inhibitor, EPZ015666 (161). Thus, for targeting FOXP3 methylation in Treg cells, it may rationalize to use SAM-competitive inhibitors. Likewise, Foxp3 associates with PRMT1 and is asymmetrically di-methylated at R48 and R51 positions. FOXP3 methylation and its function is compromised by administration of PRMT1/6 inhibitor MS023 (162). Based on the current findings, more research is needed to clarify the biological significance of the asymmetric methylation on FOXP3 in modulating Treg suppression.

IN SUMMARY

Both lymphoid-organ Treg cells and tissue-resident Treg cells require the expression of the Foxp3 transcription factor. Foxp3 protein functions in transcriptional molecule complexes. Ensemble formation is regulated by various interactions and post-translational modifications (PTMs), including phosphorylation, O-GlcNAcylation, acetylation, ubiquitination, and methylation. Modifications influence each other to orchestrate Foxp3 activity and Treg suppression. However, the currently available, published results are still not sufficient to fully reveal roles of Foxp3 PTMs in modulating Treg suppression, and some caveats should be considered when interpreting the data. For example, to what extent does the ectopic expression of protein in the transfected cell line represent the real situation in Treg cells? Are there potential substrates other than Foxp3 responsible for the phenotypes observed in those enzyme-deficient mice? What are the possible cross-talks among different Foxp3 PTMs under certain tissue- microenvironment? What is the clinical relevance and significance of Foxp3 PTMs? Moreover, are there other types of post-translational modifications occurring on Foxp3 that have not been identified yet, such as lysine crotonylation or succinylation?

The understanding of Foxp3 protein PTMs in modulating Treg suppression may facilitate the design of rational therapies for immune disorders developed in IPEX patients. Based on the high-resolution crystal structure of Foxp3, combination therapy by targeting Foxp3 post-translational modifications with other therapies may enhance therapeutic approaches in a variety of diseases. Moreover, a full understanding of the contribution of other regulatory cells, such as Suppressor T cells, may lead to additional therapeutic alternatives (163–165).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The NF-κB RelA Transcription Factor Is Critical for Regulatory T Cell Activation and Stability

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Regulatory T cells (Tregs) play a major role in immune homeostasis and in the prevention of autoimmune diseases. It has been shown that c-Rel is critical in Treg thymic differentiation, but little is known on the role of NF-κB on mature Treg biology. We thus generated mice with a specific knockout of RelA, a key member of NF-κB, in Tregs. These mice developed a severe autoimmune syndrome with multi-organ immune infiltration and high activation of lymphoid and myeloid cells. Phenotypic and transcriptomic analyses showed that RelA is critical in the acquisition of the effector Treg state independently of surrounding inflammatory environment. Unexpectedly, RelA-deficient Tregs also displayed reduced stability and cells that had lost Foxp3 produced inflammatory cytokines. Overall, we show that RelA is critical for Treg biology as it promotes both the generation of their effector phenotype and the maintenance of their identity.

Keywords: regulatory T cells, NF-κB, autoimmunity, stability, activation, relA

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INTRODUCTION

$CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) play a critical role in immune homeostasis and in the prevention of autoimmune diseases by regulating immune responses (1). In humans and mice, it is well established that *forkhead box protein 3* (*Foxp3*) deficiency conducts to the development of an autoimmune syndrome leading to early death. Although *Foxp3* plays a critical role in the differentiation, suppressive function and stability of Tregs, other transcription factors (TFs), some of which interacting with Foxp3 in multi-molecular complexes, are also involved in different aspects of their biology. Some, such as c-Rel, are involved in Treg differentiation (2, 3). Others, such as NFAT, RunX1, BACH2, or Eos are critical to maintain their suppressive activity (4–7). Another group of TFs, including Blimp1, Myb, STAT3, Tbet, IRF4, Bcl6, or PPARg are involved in further differentiation of activated Tregs and in their capacity to suppress different types of immune responses (8–14). Finally, STAT5, TET, GATA3, p300/CBP, Blimp1, or Ezh2 have been shown to maintain Treg identity and stability by controlling Foxp3 transcription and epigenetics (15–20). Although it has been reported that NF-κB is able to bind to the regulatory sequence of *Foxp3* and to interact with a complex containing Foxp3 (2, 3, 21), its role in Treg biology needs to be further analyzed.

The NF-κB TFs consist of homo or heterodimeric molecules of NF-κB1 (p105/50), RelA (p65) and c-Rel subunits for the canonical pathway and of NF-κB2 (p100/52) and RelB subunits for the

non-canonical pathway. It has been reported that c-Rel is essential for thymic Treg development by binding to the promoter sequence and the conserved non-coding sequence (CNS) 3 of *Foxp3* (2, 3, 22). The role of NF-κB in mature Treg biology has been addressed by knocking-out upstream activators of the pathway, such as IKKα and IKKß kinases. Mice with a conditional knockout (KO) in Tregs of either Ubc13, an E2 ubiquitin ligase activating IKKβ, or of IKKβ itself, develop a spontaneous autoimmune syndrome, associated with conversion of Tregs into effector-like T cells without Foxp3 loss or reduced Treg survival, respectively (23, 24). Mice with a conditional KO of IKKα in $CD4^+$ T cells have a decreased proportion of Tregs in lymphoid organs, which seem to have a defective suppression and proliferation capacities *in vivo* (25). The specific role of RelA in Tregs, which is considered as the main factor of NF-κB members in conventional T cells (26), has been recently studied. By interacting with RelA and other TFs, such as Helios and p300, Foxp3 forms a multimolecular complex localized in active nuclear areas to act primary as a transcriptional activator (27). Mice with a conditional KO of RelA in Tregs develop a severe and early spontaneous autoimmune syndrome that is associated with a defect of effector Tregs (28–30). Here, we confirmed these latter findings and added further information on the nature of the disease with extensive description of lymphoid and myeloid cell activation in lymphoid and non-lymphoid tissues. Importantly, we revealed that RelA-deficient Tregs were unstable, lost Foxp3 expression and produced inflammatory cytokines, highlighting that RelA is also critical to maintain Treg stability and identity.

RESULTS

Conditional Ablation of RelA in Tregs Leads to the Development of a Spontaneous Autoimmune Syndrome

To assess the role of RelA in Treg biology, we generated $Foxp3^{Cre}$ $Rela^{lox}$ mice that have a specific deletion of RelA in Tregs by crossing mice expressing CRE in Tregs with mice expressing a *Rela* floxed allele. In these $Foxp3^{Cre}$ $Rela^{lox}$ mice, Tregs expressed a non-functional truncated form of RelA (**Figure 1A**), as expected using this floxed allele (31). From 5 to 10 weeks of age, $Foxp3^{Cre}$ $Rela^{lox}$ mice developed a spontaneous disease characterized by localized alopecia and skin lesions (epidermal hyperplasia, hyperparakeratosis, cystic hair), and reduced weight gain compared to $Foxp3^{Cre}$ control mice (**Figures 1B,C**). This pathology had high penetrance and was severe since most of the animals had to be sacrificed for ethical reasons by 45 weeks of age (**Figures 1D,E**). At 10–12 weeks of age, $Foxp3^{Cre}$ $Rela^{lox}$ mice exhibited adenomegaly and macroscopic signs of mild colon inflammation (**Figures 1F,G**). Histological analyses showed moderate immune cell infiltration in the lung, stomach and colon and high level of immune cell infiltration in the skin (**Figure 1H**). The liver and small intestine were not or minimally infiltrated. Thus, mice with RelA-deficient Tregs developed a severe and systemic inflammatory syndrome.

We started the characterization of this syndrome by analyzing the lymphocyte compartment of 10–12 week-old $Foxp3^{Cre}$ $Rela^{lox}$ mice. Numbers of $CD45^+$ leukocytes were highly increased in the skin draining lymph nodes (sdLN), the internal LN (iLN, corresponding to pancreatic and paraaortic LN) and the inflamed non-lymphoid tissues (lung and skin) but not in the spleen, mesenteric LN (mLN) or the non-inflamed non-lymphoid tissues (liver, small intestine) (**Figure 2A**). This leukocyte expansion was due to increased numbers of $CD8^+$ and $CD4^+$ T cells, B cells (**Figure 2B** and data not shown) and myeloid cells (see below). Moreover, the proportions of $CD44^{high}CD62L^{low}$, $ICOS^+$, and $Ki67^+$ activated/memory $CD8^+$ and $CD4^+$ conventional T cells were significantly increased in the spleen, sdLN, and lung of $Foxp3^{Cre}$ $Rela^{lox}$ mice compared to $Foxp3^{Cre}$ control mice (**Figures 2C,D** and **Supplementary Figure 1A**). The same tendency was observed in the colon and skin, although this was not significant, probably because basal levels of activated cells were already high in $Foxp3^{Cre}$ control mice. Interestingly, an increased proportion of activated/memory T cells was observed in the iLN and mLN as well as in the non-inflamed liver and small intestine, demonstrating a global systemic T cell activation in $Foxp3^{Cre}$ $Rela^{lox}$ mice (**Supplementary Figure 1B**). Systemic inflammation was confirmed by quantifying cytokines in the serum, where we observed highly increased levels of IFNγ, IL-4, IL-10, IL-17, IL-6, and TNFα (**Figure 2E**). Also, serum levels of IgM, IgG1, IgG2b, IgA, and IgE (**Figure 2F**) and of anti-DNA autoantibodies (**Figure 2G**) were increased in 12–14 week-old sick $Foxp3^{Cre}$ $Rela^{lox}$ mice compared to $Foxp3^{Cre}$ control mice.

The systemic inflammation was further documented by analyzing myeloid cells, characterized as shown in **Supplementary Figure 2A**. Their numbers were strongly increased in the spleen and sdLN as well as in the inflamed non-lymphoid tissues, lung and skin, in $Foxp3^{Cre}$ $Rela^{lox}$ mice compared to controls (**Supplementary Figure 2B**). This increase of myeloid cells was due to an increase of neutrophils in all these tissues and of eosinophils and monocytes in the lymphoid organs and the skin (**Supplementary Figure 2C**). A similar trend was observed in the colon.

Only part of this inflammatory phenotype was observed in 4–6 week-old $Foxp3^{Cre}$ $Rela^{lox}$ mice. Increased numbers of whole $CD45^+$ leukocytes were observed in sdLN and iLN but not yet in the lung and skin (**Supplementary Figure 3A**). A trend for higher proportion of activated/memory T cells, defined by expression of CD44, CD62L and Ki67, was observed in all analyzed lymphoid and non-lymphoid tissues of young mice (**Supplementary Figure 3B**). Finally, inflammatory cytokines, natural antibodies and anti-DNA antibodies were not or minimally increased in 4–6 week-old $Foxp3^{Cre}$ $Rela^{lox}$ compared to control mice (**Figures 2E–G**). In conclusion, $Foxp3^{Cre}$ $Rela^{lox}$ mice developed a severe systemic autoimmune syndrome, already uncovered at 4–6 weeks of age, followed, 1–3 months later, by massive activation of T cells, immune infiltration of several tissues and high rise of serum inflammatory cytokines, immunoglobulins, and auto-antibodies.

Tregs of $Foxp3^{Cre}$ $Rela^{lox}$ Mice Appear to Be Less Stable

We then analyzed Treg homeostasis in 12 week-old $Foxp3^{Cre}$ $Rela^{lox}$ mice. Strikingly, Treg proportion was significantly increased in lymphoid organs, except in mLN, while it was decreased in the colon and skin and unchanged in the liver,

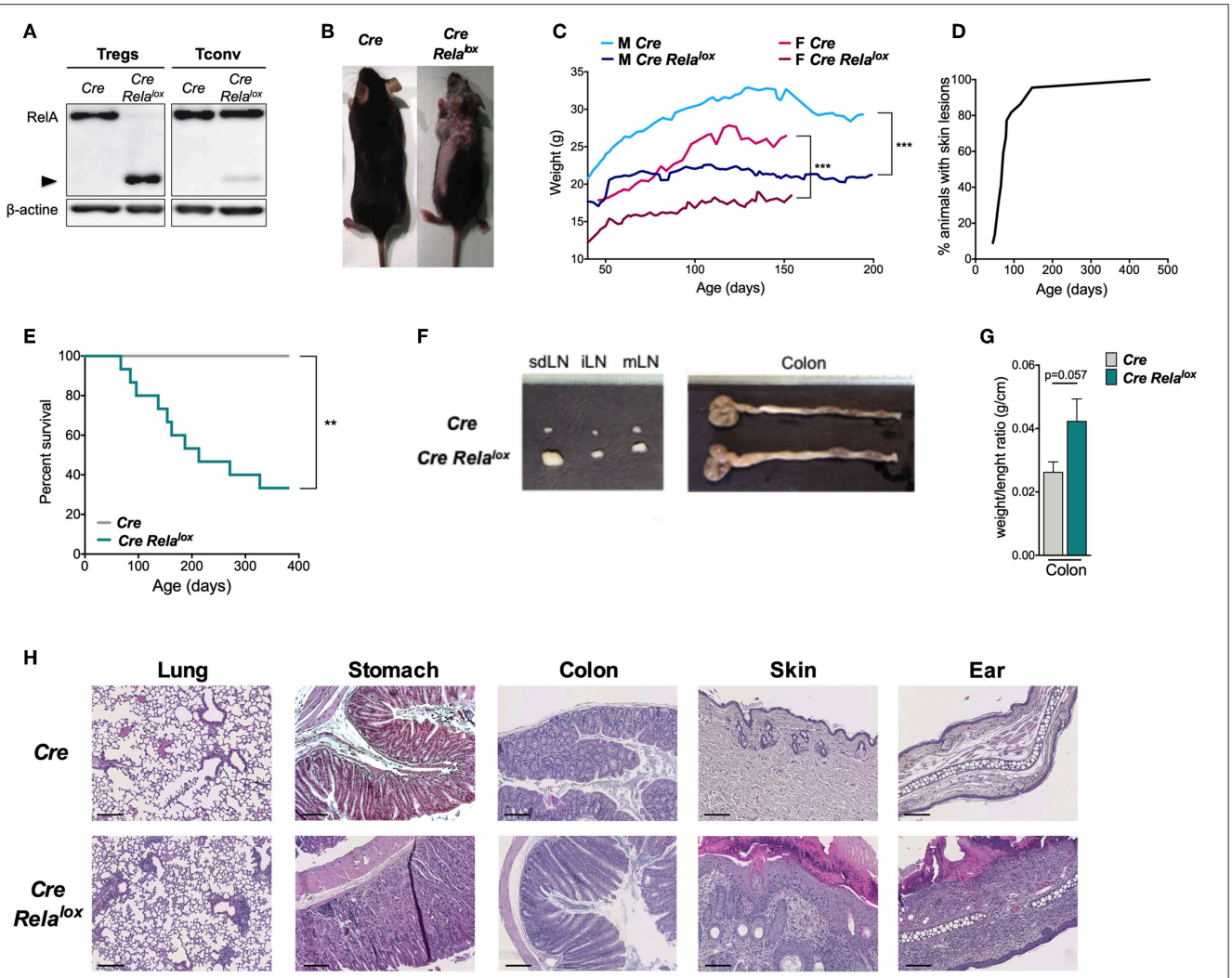

FIGURE 1 | Mice with RelA deficient Tregs develop systemic inflammation. **(A)** Western blot analysis of RelA expression in Tregs and CD4$^+$ conventional T cells (Tconv) isolated from *Foxp3*^{Cre} (*Cre*) and *Foxp3*^{Cre} *Rela*^{lox} (*Cre Rela*^{lox}) mice. **(B)** Representative pictures of 12 week-old *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice. **(C)** Body weight monitoring of *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} males and females. **(D)** Percentages of *Foxp3*^{Cre} *Rela*^{lox} mice with skin lesions. **(E)** Survival monitoring of *Foxp3*^{Cre} *Rela*^{lox} mice. **(F)** Representative pictures from 20 mice of the LN and colon of 12 week-old *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice. **(G)** Weight/length ratio of colon of 12 week-old *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice. **(H)** Representative histology from 12 week-old mice of the lung, stomach, colon, skin, and ear of *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox}. Scale bars represent 200 μm (lung, *Foxp3*^{Cre} stomach, colon), 150 μm (*Foxp3*^{Cre} *Rela*^{lox} stomach), and 100 μm (skin, ear). Data are representative of independent experiments. Bars show the means and error bars represent SEM. For mouse and experiment numbers, see **Supplementary Table 1**. Statistical significance was determined using a log-rank (Mantel- Cox) test for the mouse survival data. The two-tailed unpaired non-parametric Mann–Whitney *U*-test was used. $^{**}p < 0.01$, $^{***}p < 0.001$.

lung and small intestine compared to *Foxp3*^{Cre} control mice (**Figure 3A**). Interestingly, in the small intestine, colon and skin of 5 week-old *Foxp3*^{Cre} *Rela*^{lox} mice, Treg proportion and number (except in the skin) seemed already decreased, when compared to 12 week-old *Foxp3*^{Cre} *Rela*^{lox} mice (**Figure 3B**; **Supplementary Figure 4**). The proportion of activated/memory CD44^{hi}CD62L^{low} Tregs was decreased in all LN and the liver, and the same tendency was observed in the skin. However, their proportion was unchanged in the spleen, colon and small intestine and even increased in the lung (**Figure 3C**). Foxp3 and CD25 expressions were unchanged (data not shown).

The severe disease of *Foxp3*^{Cre} *Rela*^{lox} mice in the absence of major Treg quantitative defect suggests that Tregs may be dysfunctional. *In vitro* assays showed that RelA-deficient Tregs, purified from 5 to 6 week-old mice, were able to suppress proliferation of conventional T cells almost as efficiently as control Tregs (**Figure 3D**). To further analyze their function, we assessed their capacity to suppress colitis induced by effector T cells transferred into lymphopenic mice, measured by weight loss and histology. Surprisingly, not only RelA-deficient Tregs were unable to control colitis but the disease was even more severe compared to mice transferred with effector T cells

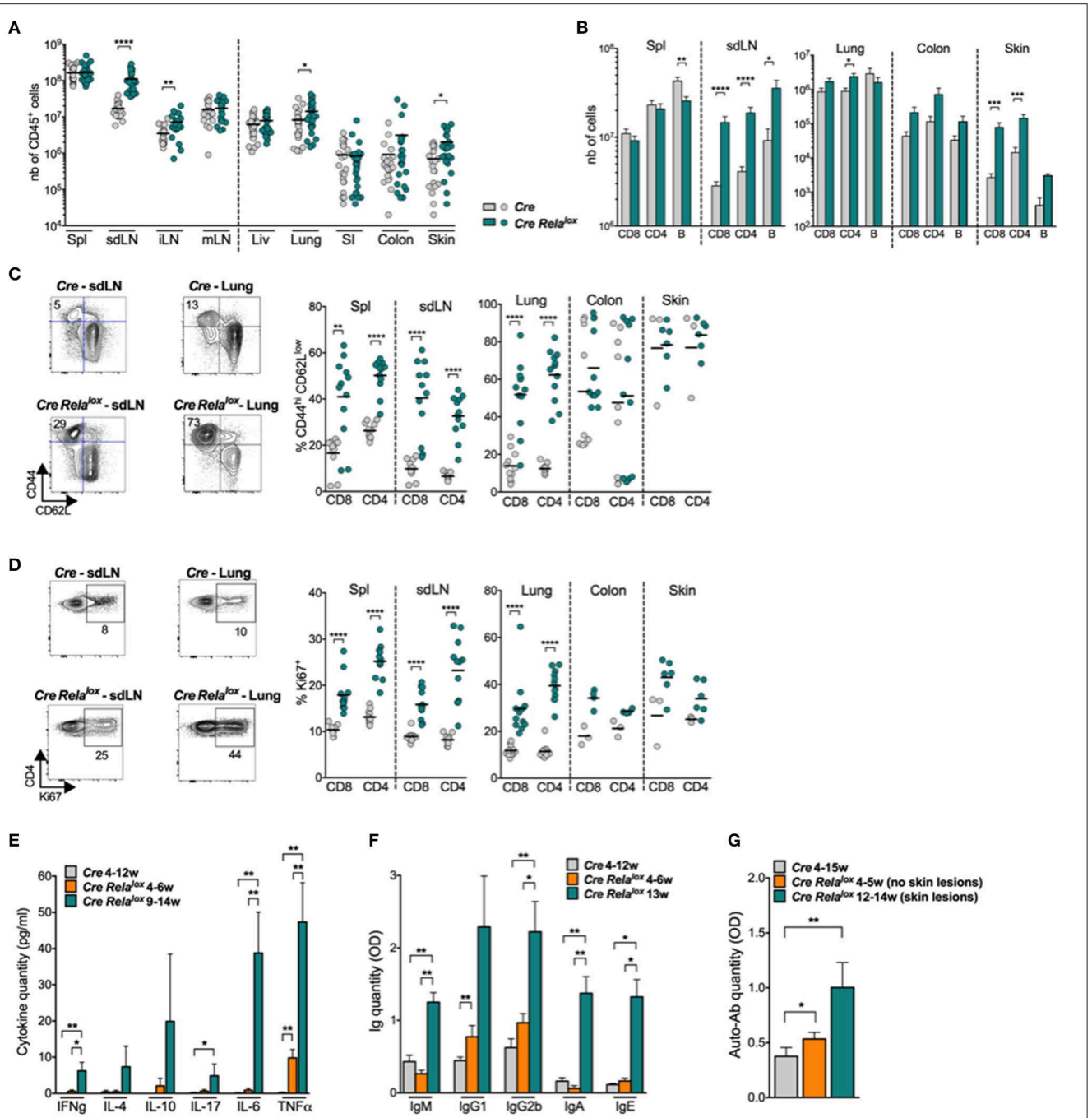

FIGURE 2 | High activation of T and B lymphocytes in *Foxp3*^{Cre} *Rela*^{lox} mice. **(A,B)** Number of CD45^{+} **(A)**, CD8^{+}, CD4^{+}, and B cells **(B)** in the indicated organs (spl, spleen; liv, liver; SI, small intestine) of 12 week-old *Foxp3*^{Cre} (*Cre*) and *Foxp3*^{Cre} *Rela*^{lox} (*Cre Rela*^{lox}) mice. **(C,D)** Representative dot plots and proportion of CD44^{hi} CD62L^{low} **(C)** and Ki67^{+} **(D)** among CD8^{+} and CD4^{+} Tconv in the indicated organs of 12 week-old *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice. **(E)** Cytokine quantification in the serum of 4–12 week-old *Foxp3*^{Cre}, and 4–6 week-old and 9–14 week-old *Foxp3*^{Cre} *Rela*^{lox} mice. **(F)** Immunoglobulin quantification in the serum of 4–12 week-old *Foxp3*^{Cre} mice, and 4–6 week-old and 13 week-old *Foxp3*^{Cre} *Rela*^{lox} mice. **(G)** Anti-DNA antibody quantification in the serum of 4–15 week-old *Foxp3*^{Cre} mice, and 4–12 week-old and 12–14 week-old *Foxp3*^{Cre} *Rela*^{lox} mice. Each dot represents a mouse, lines and bars show the means of pooled independent experiments. Error bars represent SEM. For mouse and experiment numbers, see **Supplementary Table 1**. The two-tailed unpaired non-parametric Mann–Whitney *U*-test was used for data not following a normal distribution and the *t*-test was used for data following a normal distribution. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

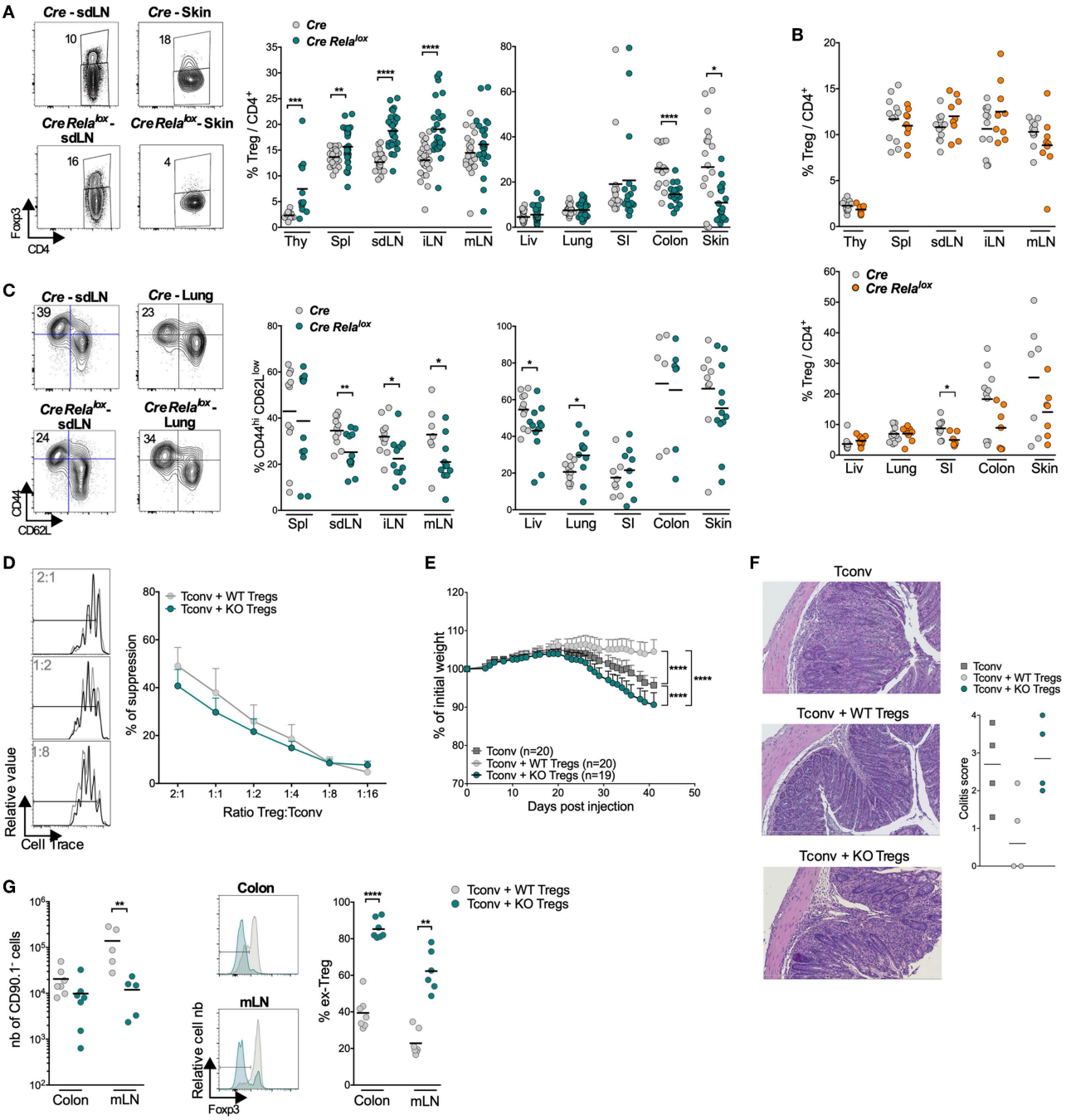

FIGURE 3 | Tregs in *Foxp3*^{Cre} *Rela*^{lox} mice appear to be less stable. (A) Representative density plot and proportion of Tregs among the CD4^{+} T cells in the indicated organs (thy, thymus; spl, spleen; liv, liver; SI, small intestine) of 12 week-old *Foxp3*^{Cre} (*Cre*) and *Foxp3*^{Cre} *Rela*^{lox} (*Cre Rela*^{lox}) mice. **(B)** Proportion of Tregs among CD4+ cells in 5 week-old *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice. **(C)** Representative density plots and proportions of CD44^{hi} CD62L^{low} among the Tregs of 12 week-old *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice. Each dot represents a mouse and lines show the means of pooled independent experiments. **(D)** *In vitro* suppressive activity of Treg cells from *Foxp3*^{Cre} (WT Tregs) and *Foxp3*^{Cre} *Rela*^{lox} (KO Tregs) 5–6 week-old mice. Representative data at 2:1, 1:2 and 1:8 (left) and different (right) Treg:Tconv ratios of independent experiments. **(E–G)** *In vivo* suppressive activity of Treg cells from *Foxp3*^{Cre} (WT Tregs, 6 week-old mice) and *Foxp3*^{Cre} *Rela*^{lox} (KO Tregs, 6 week-old mice) mice, determined in a colitis model stopped at 6 weeks for analyses. **(E)** Percentage of initial body weight pooled from independent experiments. Error bars represent SEM. **(F)** Representative histology of the colon and colitis scores. **(G)** Numbers of recovered Tregs (CD90.1^{-} cells), representative histograms and proportions of ex-Treg in the mLN and colon. Each dot represents a mouse and lines show the means of pooled independent experiments. For mouse and experiment numbers, see **Supplementary Table 1**. The two-tailed unpaired non-parametric Mann–Whitney *U* test was used for data not following a normal distribution and the *t*-test was used for data following a normal distribution. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

alone (**Figures 3E,F**). This exacerbated colitis was not associated with increased number of cells from Tconv origin (CD90.1$^+$ cells) or to their lower propensity to differentiate in peripheral Treg (pTregs) (**Supplementary Figure 5**). Instead, the severe colitis was rather due to the fact that most RelA-deficient Tregs lost Foxp3 expression in the colon and mLN, potentially differentiating in pathogenic effector T cells (**Figure 3G**). In conclusion, *Foxp3^{Cre} Rela^{lox}* mice had higher numbers of Tregs in lymphoid tissues (probably due to systemic inflammation) but lower numbers of Tregs in the colon and skin, which could be due to Treg instability and Foxp3 loss.

RelA Deficiency Leads to a Defect of Effector Tregs at Steady State

Foxp3^{Cre} Rela^{lox} mice developed systemic inflammation, which in return impacted on Treg biology. Thus, to assess the intrinsic role of RelA in Tregs at steady state, we generated *Foxp3$^{Cre/wt}$ Rela^{lox}* heterozygous females, in which theoretically half of Tregs expressed RelA and the other half were RelA-deficient because of the localization of *Foxp3* locus in the X chromosome. We observed that these mice did not have any sign of disease and inflammation, as first noticed by macroscopic observations and the absence of cell infiltration in tissues (**Figures 4A,B**), which was most likely due to the presence of functional RelA-sufficient Tregs. This was further confirmed by analyzing the numbers of CD45$^+$ leukocytes and Tregs that were similar in *Foxp3$^{Cre/wt}$ Rela^{lox}* females and *Foxp3$^{Cre/wt}$* controls (**Figures 4C,D**). Moreover, the proportions of activated conventional T cells (Tconvs), defined by the expression of CD44, CD62L and Ki67, was identical between the two mouse types (**Figure 4E**). Finally, no increased level of anti-DNA auto-antibodies were detected in the serum of the *Foxp3$^{Cre/wt}$ Rela^{lox}* females (**Figure 4F**). Thus, *Foxp3$^{Cre/wt}$ Rela^{lox}* heterozygous females represent a proper model to study the intrinsic role of RelA in Tregs.

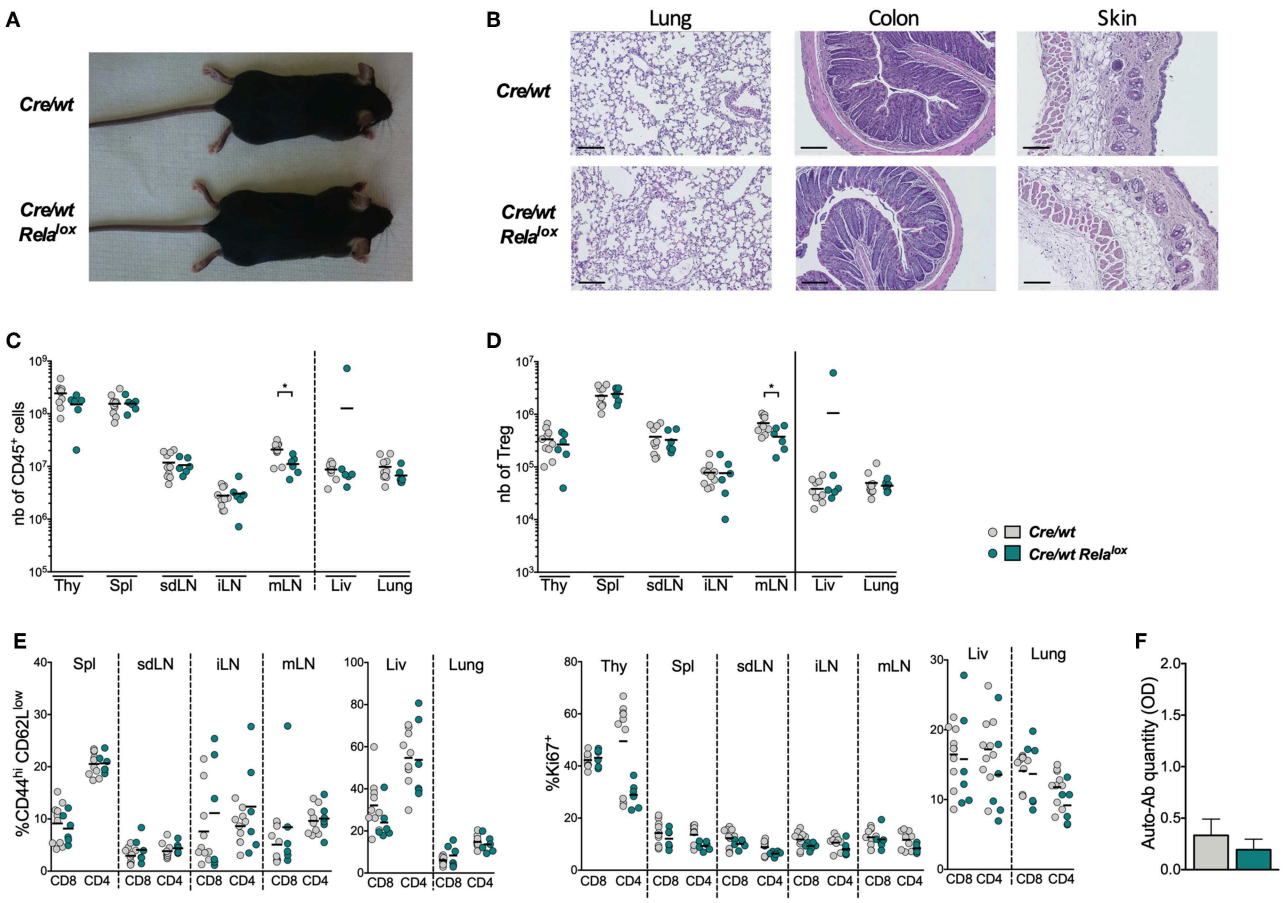

FIGURE 4 | Heterozygous *Foxp3$^{Cre/wt}$ Rela^{lox}* do not develop systemic inflammation. **(A)** Representative pictures of 8 week-old mice. **(B)** Representative histology of lung, colon and skin of 8 week-old. Scale bars represent 100 μm. Number of CD45$^+$ **(C)**, of Tregs among CD4$^+$ T cells **(D)**, and proportion of CD44^{hi}CD62L^{low} and Ki67$^+$ among CD8$^+$ and CD4$^+$ conventional T cells **(E)** in different tissues (thy, thymus; spl, spleen; liv, liver; SI, small intestine) of 8 week-old *Foxp3$^{Cre/wt}$* (*Cre/wt*) and *Foxp3$^{Cre/wt}$ Rela^{lox}* (*Cre/wt Rela^{lox}*) mice. Each dot represents a mouse and lines show the means of pooled independent experiments. **(F)** Anti-DNA auto-antibodies quantification in the serum of 8 week-old *Foxp3$^{Cre/wt}$* and *Foxp3$^{Cre/wt}$ Rela^{lox}* mice. Bars show the means of pooled independent experiments and error bars represent SEM. For mouse and experiment numbers, see **Supplementary Table 1**. The two-tailed unpaired non-parametric Mann–Whitney *U* test was used *$p < 0.05$.

In the *Foxp3*$^{Cre/wt}$ control females, CRE-expressing Tregs (CRE$^+$) were present in lower proportion compared to Tregs not expressing CRE (CRE$^-$) (**Figure 5A**, gray bars). The same tendency was observed for the different molecules that we investigated (**Figures 5B–E**, gray bars), suggesting that the CRE transgene impacts on Treg biology in this competitive condition. Compared to these controls, the knockout of RelA did not modify significantly the proportion of Tregs (**Figure 5A**, green bars) nor the proportion of Tregs expressing ICOS, CTLA-4, Nrp1 or Helios (**Supplementary Figure 6**). However, the absence of RelA expression had a severe impact on Treg activation since the proportions of CD44^{high}CD62L^{low}, Ki67$^+$, CD103$^+$ and the expression level of GITR among CRE$^+$ Tregs were strongly and systematically reduced (**Figures 5B–E**). In conclusion, RelA expression by Tregs appears critical for the acquisition of their effector phenotype at the steady state.

RelA Plays an Important Role in Treg Activation

To characterize more extensively the effects of the RelA deficiency on Tregs, we purified CRE-expressing Tregs from *Foxp3*$^{Cre/wt}$ (WT) and *Foxp3*$^{Cre/wt}$ *Rela*^{lox} (RelA KO) mice and profiled their transcriptomes by low-input RNAseq. Overall, transcriptome differences were modest (**Figures 6A,B**), with 180 differentially expressed genes at an arbitrary fold change cutoff of 2.0 (and false discovery rate < 0.05). The most biased transcript was *Klrg1*, as previously reported (28), but several other transcripts involved in Treg function and/or homing in the gut and skin showed a significant bias (e.g., *Ccr4, Ccr6, Maf, Ahr*, and *Itgae*) (**Figures 6B,C**). Gene ontology analysis did not reveal any evocative common pathway, so we projected various Treg-specific signatures onto the comparison of WT vs. RelA KO Tregs profiles (**Figure 6D**). RelA deficit modestly but significantly affected Treg identity as it reduced the canonical signature of genes differentially expressed in Tregs compared to Tconv cells (33) (**Figure 6D**, left). Moreover, consistent with the phenotype described above showing reduced proportion of activation markers in RelA-deficient Tregs in *Foxp3*$^{Cre/wt}$ *Rela*^{lox} mice, a stronger bias was observed for signatures typical of activated Tregs [from comparison of CD44^{hi} vs. CD62L^{hi} Tregs, or from Blimp1- WT vs. KO Tregs (11)]. Indeed, RelA-deficient Tregs had a transcriptional signature analogous to CD62L^{hi} Tregs and Blimp1 KO Tregs, corresponding to resting-like Tregs (**Figure 6D**, middle and right). This effect was not unique to activated Treg signature, as GSEA analysis showed a strong bias of generic signatures of activated CD4$^+$ or CD8$^+$ Tconv cells (32) (**Figure 6E**). For further resolution, we cross-matched the RelA WT/KO difference to a curated series of 289 signatures that distinguish different sub-phenotypes of Tregs (34) (**Figure 6F**). The enrichment score of several gene sets characterizing activated or effector Tregs were decreased in RelA KO Tregs compared to WT Tregs (lower region of **Figure 6F**). Interestingly, however, RelA-deficient Tregs were enriched in several signatures resulting from the expression of TF with inhibitory roles in Tregs, and most markedly for Bach2 (upper region of **Figure 6F**). Indeed, the changes found here in response to RelA deficiency were largely anti-correlated with changes provoked by the absence of Bach2 in a previous report (7) (**Figure 6G**, $r = -0.13$ with $p < 10^{-15}$ using a Pearson correlation). Overall, compared to WT Tregs, the transcriptomic signature of RelA-deficient Tregs confirmed their resting phenotype.

RelA-Deficient Tregs Have a Defect of Stability

Our RNAseq data indicate an identity defect of RelA-deficient Tregs, which was first suggested in the colitis model (**Figures 3E–G**). However, one cannot conclude from this latter experiment that RelA plays an intrinsic role in Treg stability, owing to the very severe colitis developed by the mice injected with RelA-deficient Tregs. Indeed, increased instability of these latter could be well due to increased inflammation, and not RelA deficiency, since it is well established that different inflammatory factors precipitate Foxp3 loss (35). Thus, we further investigated whether RelA had any role in maintenance of Treg stability and identity by analyzing Foxp3 expression after co-transfer of RelA-sufficient and -deficient Tregs into the same mouse. Cells were purified from *Foxp3*$^{Cre/wt}$ *Rela*^{lox} mice (*Foxp3*$^{Cre/wt}$ for controls) and not from *Foxp3*^{Cre} *Rela*^{lox} mice, since systemic inflammation in these latter mice could modify Treg biology in addition to the impact of the RelA defect. Tregs were co-transferred in CD3 KO mice with Tconvs to sustain viability and expansion of injected Tregs (**Figure 7A**). Sixteen days after transfer, the proportions of RelA-deficient cells were much lower than the ones of RelA-sufficient cells (**Figure 7B**), particularly in the colon, a location subjected to high inflammation in this setting. Importantly, a large fraction of RelA-deficient Tregs lost Foxp3 expression, becoming so-called ex-Tregs, in all lymphoid and non-lymphoid tissues, compared to RelA-sufficient Tregs (**Figure 7C**). Moreover, RelA-deficient ex-Tregs expressed higher amounts of the pro-inflammatory cytokines IFNγ and TNFα, in the spleen and mLN, than their wildtype counterparts (**Figure 7D**).

To further explore the mechanism of Treg instability, and since it has been reported that c-Rel is involved in the differentiation of Th1 and Th17 cells (36, 37), we performed electrophoretic mobility shift assays (EMSA) combined with supershifts to assess the activation status of the different NF-κB subunits in Tregs of *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice (**Figure 7E**). In control Tregs, there was mainly an activation of RelA, rather than RelB or c-Rel. As expected, we did not observe this phenomenon in RelA-deficient Tregs, confirming that the truncated RelA protein was not functional. However, in Tregs of *Foxp3*^{Cre} *Rela*^{lox} mice there were much more activated NF-κB complexes, obviously due to the more activated phenotype of Tregs in these mice, which were mostly, if not only, constituted of c-Rel subunit. This massive c-Rel activation may be involved in Treg instability. In conclusion, our data show that lack of RelA activation strongly affect Treg stability leading to Foxp3 loss and increased differentiation of ex-Tregs, which may turn pathogenic through the production of inflammatory cytokines.

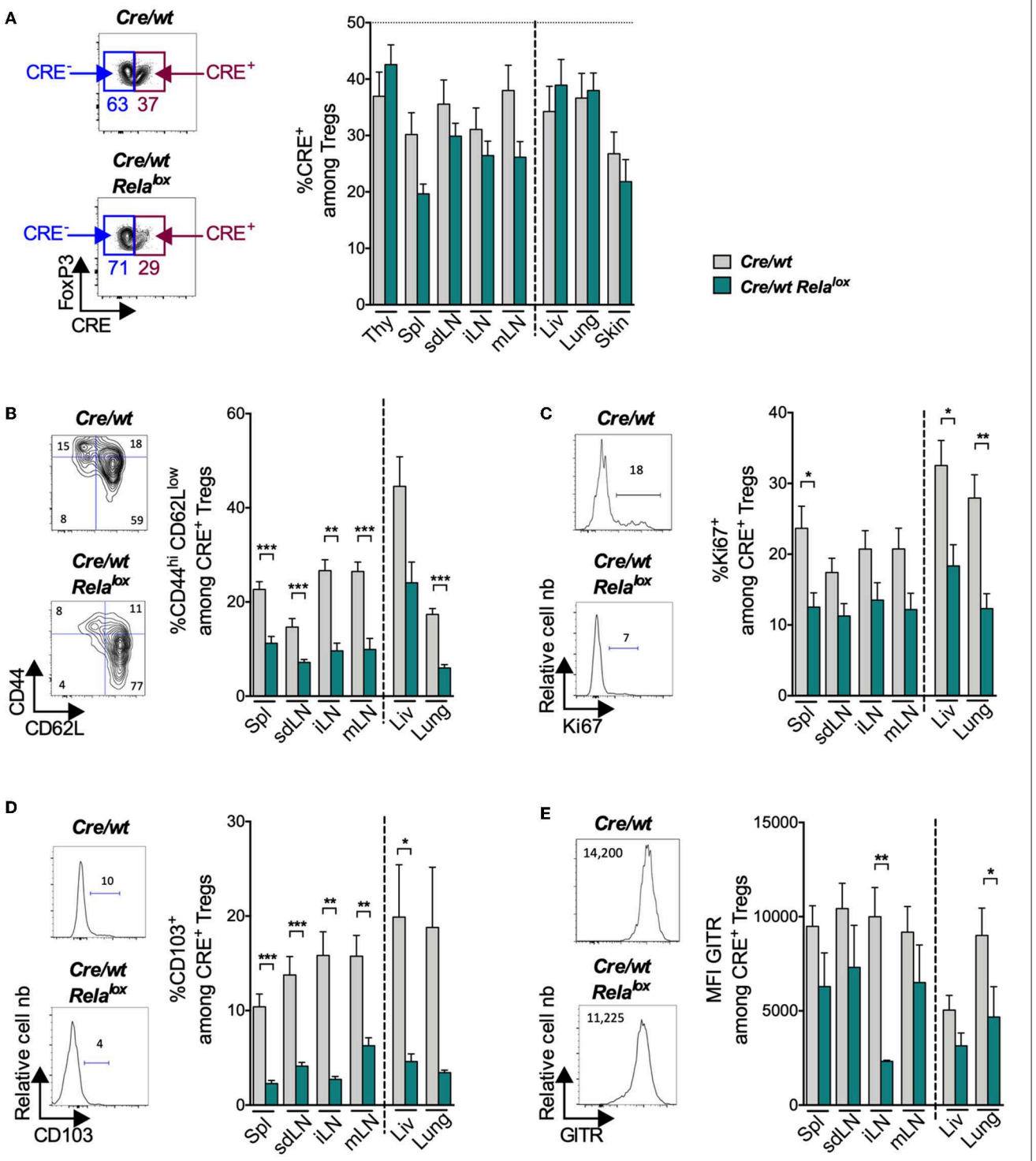

FIGURE 5 | Reduced expression of activation markers in RelA-deficient Tregs at steady state. Analyses in the indicated organs (thy, thymus; spl, spleen; liv, liver) of 8 week-old *Foxp3*$^{Cre/wt}$ (*Cre/wt* – gray bars) and *Foxp3*$^{Cre/wt}$ *Rela*^{lox} (*Cre/wt Rela*^{lox}–green bars) mice. **(A)** Representative density plots among CD4^{+} cells to define Tregs expressing CRE (CRE^{+}) and percentages of CRE^{+} among total Tregs in sdLN. Representative density plots and proportions of CD44^{hi} CD62L^{low} **(B)**, Ki67^{+} **(C)**, CD103^{+} **(D)** and MFI of GITR **(E)** among CRE^{+} Tregs of sdLN. Bars show the means of pooled independent experiments and error bars represent SEM. For mouse and experiment numbers, see **Supplementary Table 1**. The two-tailed unpaired nonparametric Mann–Whitney *U*-test was used. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

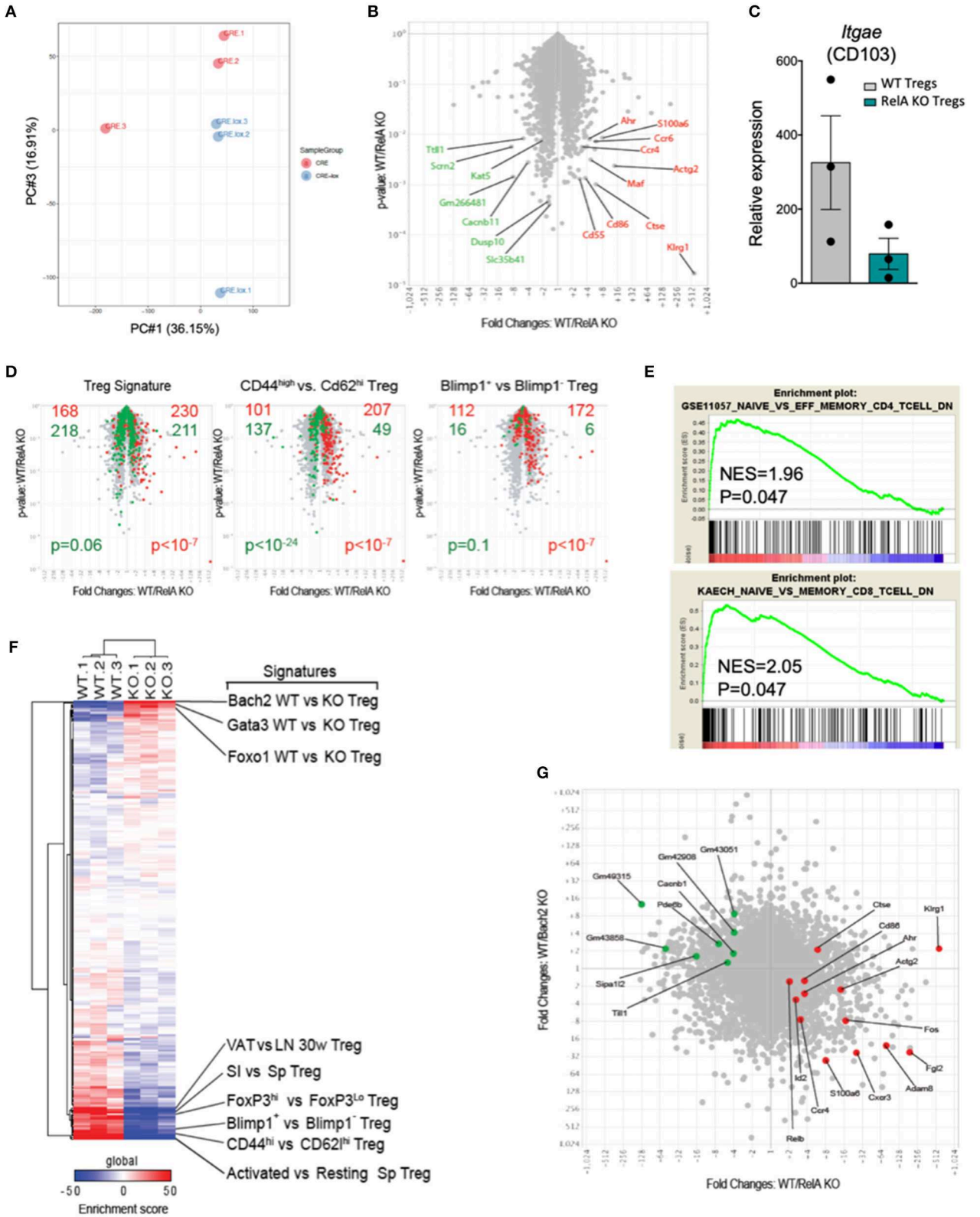

FIGURE 6 | RelA-deficient Tregs have identity and activation defects. **(A)** PCA analysis of WT and RelA KO Tregs. **(B)** Volcano plot of WT vs. RelA KO Tregs. Red and green indicate transcripts up- and down-regulated, respectively, by WT Tregs cells. **(C)** Relative expression of *Itgae* (CD103) expressed in counts per million in WT and RelA KO Tregs. **(D)** WT vs. RelA KO Tregs (as in **A**) overlaid with various Tregs signatures. Red and green indicate genes up- and down-regulated, respectively, in each

(Continued)

FIGURE 6 | signature (chi-squared test for *p*-value). **(E)** GSEA plots of RelA-deficient Tregs compared with indicated set of genes up-regulated in effector memory CD4 (upper panel) and memory CD8 conventional T cells (lower panel) (32). **(F)** Heatmap for the enrichment score of each gene signature (VAT, visceral adipose tissue; LN, lymph nodes; SI, small intestine; Sp, spleen). **(G)** Fold change-fold change plot of WT vs. RelA KO Tregs (x-axis) and WT iTregs vs. WT Bach2 KO iTregs [y-axis, from published data (7)]. Red and green transcripts from **(A)**. For mouse and experiment numbers, see **Supplementary Table 1**.

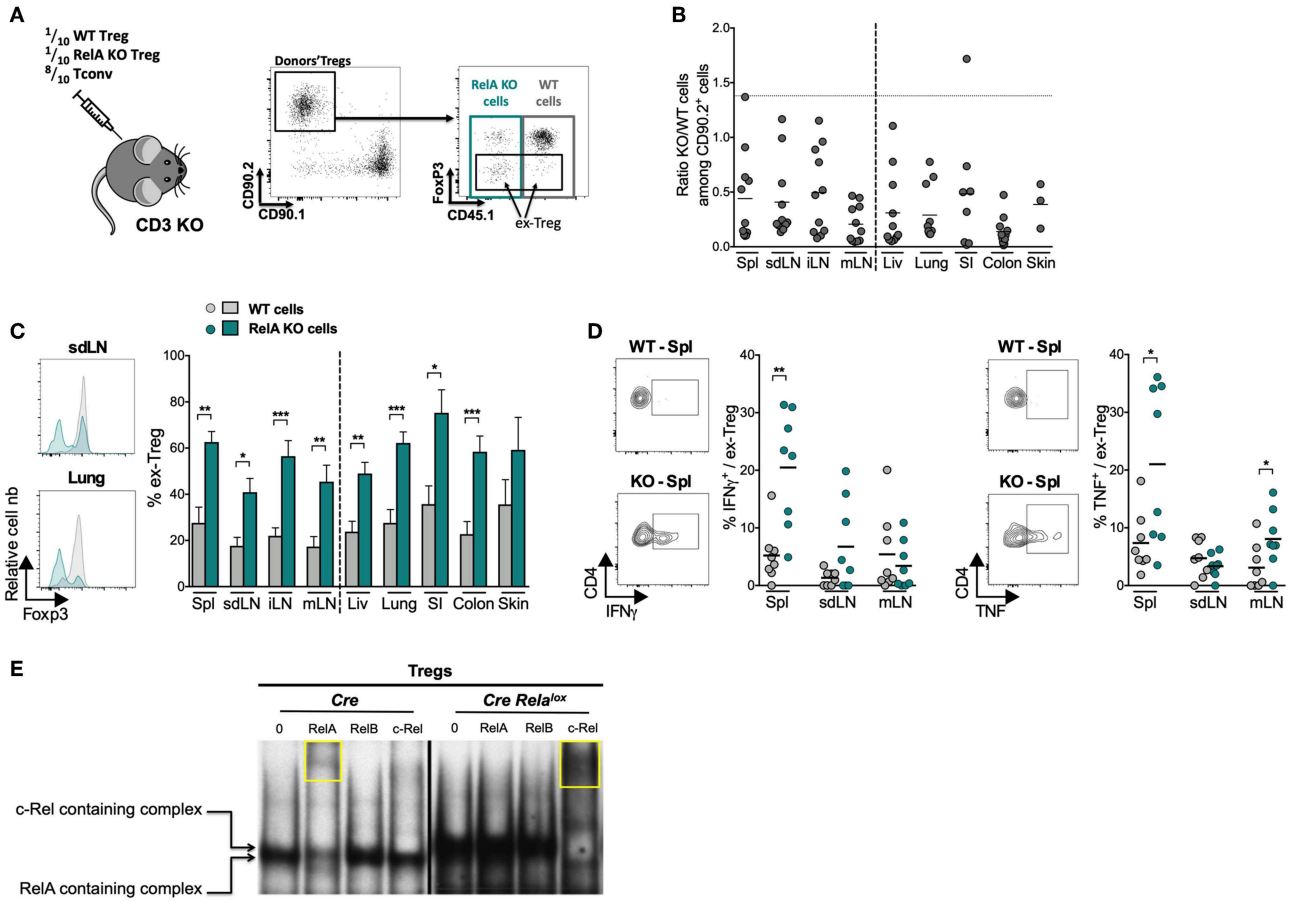

FIGURE 7 | RelA-deficient Tregs are unstable and turn pathogenic. **(A–D)** Adoptive transfer of a 1:1:8 ratio of a mix of CRE-expressing Tregs from *Foxp3*$^{Cre/wt}$ (CD45.1/2 CD90.2 WT Tregs), *Foxp3*$^{Cre/wt}$ *Rela*^{lox} (CD45.2 CD90.2 RelA KO Tregs) mice and CD4^{+} conventional T cells (CD90.1 Tconv) into CD3 KO mice and analysis of donor cells 16 days later. **(A)** Experimental scheme and representative gating strategy from sdLN staining. **(B)** Ratio of RelA KO to WT Tregs in the indicated organs (spl, spleen; liv, liver; SI, small intestine) among CD90.2^{+} donor cells. The horizontal dot line represents the initial ratio (in the syringe). **(C)** Representative histograms and proportion of ex-Tregs from injected WT cells and RelA KO cells in different tissues. **(D)** Representative density plots and proportions of IFNγ^{+} and TNF^{+} cells among WT ex-Tregs and RelA KO ex-Tregs. Each dot represents a mouse, lines and bars show the means of pooled independent experiments. Error bars represent SEM. **(E)** EMSA combined with supershift assay analysis of NF-κB subunits activation in Tregs isolated from *Foxp3*^{Cre} (*Cre*) and *Foxp3*^{Cre} *Rela*^{lox} (*Cre Rela*^{lox}) mice. The yellow squares point out the supershift of RelA or c-Rel containing complexes. The results are representative of independent experiments. For mouse and experiment numbers, see **Supplementary Table 1**. The two-tailed unpaired non-parametric Mann–Whitney *U*-test was used for data not following a normal distribution and the *t*-test was used for data following a normal distribution $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

Here, we show that RelA plays a major role in Treg biology, both at steady state and during inflammation, since its specific deletion leads to the development of a spontaneous, severe, and systemic autoimmune syndrome.

The disease recapitulates some of the symptoms observed in Treg-deficient scurfy mice, although with a slower kinetics (1). As in scurfy mice, the skin and lymphoid organs are the most impaired tissues of *Foxp3*^{Cre} *Rela*^{lox}, followed by the lung, stomach and colon and at lower extent the small intestine and liver. Also, we detected DNA auto-antibodies in the serum of our mice, as in scurfy mice (38, 39). We thus presume that *Foxp3*^{Cre} *Rela*^{lox} mice develop an autoimmune syndrome due to defective Tregs. Importantly, modification of the microbiota could play a major role in some tissue impairment such as the colon. Indeed, in *Foxp3*-deficient mice, colon damage becomes severe only after weaning, when microbial flora

develops extensively (38). Our data suggest that this disease is initially due to a major activation defect of RelA-deficient Tregs. Indeed, in the *Foxp3*$^{Cre/wt}$ *Rela*^{lox} non-inflamed mice, we observed reduced numbers of effector Tregs and suppressive molecules among the RelA-deficient Tregs. We thus speculate that in the *Foxp3*^{Cre} *Rela*^{lox} mice, and more specifically in tissues that are in contact with external environment and microbiota like the intestine and skin, effector T cells and myeloid cells become highly activated because of insufficient control by effector Tregs. Moreover, the decreased Treg proportion and number observed in those tissues in 5 week-old *Foxp3*^{Cre} *Rela*^{lox}, potentially due to Treg instability and a decreased expression of gut and skin homing molecules (reduced mRNA levels of *Ccr4*, *Ccr6*) (40, 41), may exacerbate this phenomenon. Then, inflammatory factors may alter drastically stability of RelA-deficient Tregs most of them becoming pathogenic ex-Tregs, as we observed in the colitis model and cell co-transfer in lymphopenic mice experiments, precipitating local inflammation. The combination of reduced Treg number in the intestine and the skin, reduced Treg activation and the generation of pathogenic ex-Tregs may be the driving forces of the autoimmune syndrome of *Foxp3*^{Cre} *Rela*^{lox} mice.

Recent reports describe similar conditional KO mice developing a related autoimmune syndrome (28–30). They observed that *Foxp3*^{Cre} *Rela*^{lox} mice developed inflammation of the skin, stomach, lung and colon, massive activation of effector T cells and myeloid cells in lymphoid organs and high levels of inflammatory cytokines, immunoglobulins and anti-DNA in the serum. We confirmed these data and got deeper into the analysis of the disease since we showed that the effector T cells and myeloid cells were also drastically activated in multiple non-lymphoid organs. These data suggest a major defect of RelA-deficient Tregs. In addition, the injection of WT Tregs before 7 days of age was sufficient to stop the development of the pathology (data not shown). Surprisingly, we and others observed an increase of Treg proportion in lymphoid organs and *in vitro* assays did not reveal Treg suppressive defect. However, our extensive analysis enabled to point out a decrease of Treg proportion in the inflamed non-lymphoid tissues, such as the colon and skin. Our RNAseq analysis revealed a decreased expression of *Ccr4, Ccr6, Maf, Ahr,* and *Itgae* (encoding for CD103) which are involved in Treg function and/or homing in those tissues. Particularly, it has been shown that *Ahr* regulates the expression of *Ccr6* and *Itgae* and that *Ahr* deletion in Tregs leads to their decrease in the gut (42). As discussed above, this initial event may ignite the whole immune system, leading to widespread activation of the lymphoid and myeloid compartments and release of inflammatory cytokines that will boost global Treg activation and expansion, which remains insufficient to control the pathology.

Investigating initial events that led to disease could not be properly analyzed in *Foxp3*^{Cre} *Rela*^{lox} mice since inflammation has major impact on Treg migration, survival, activation, suppressive function or stability (35, 43), confounding the interpretation of what was due to inflammation or to the intrinsic RelA deficit. Using *Lck*^{Cre} *Rela*^{lox} mice, Messina et al. suggested that a major alteration of RelA-deficient Tregs was their defect to differentiate in effector Tregs (28). However, in this work, this defect was only partial, observed in LN and not in the spleen, and mostly analyzed in a quite irrelevant model since RelA was knockout in whole T cells. Vasanthakumar et al. showed a more global activation defect of RelA-deficient Tregs using *Foxp3*$^{Cre/wt}$ *Rela*^{lox} mice or mixed bone marrow chimeric mice (29). We confirmed and completed these results by showing a downregulation of CD44, CD103, Ki67, and GITR not only in the lymphoid organs but also in the liver and lung of *Foxp3*$^{Cre/wt}$ *Rela*^{lox} mice. Moreover, our transcriptomic analysis highlighted the major activation defect of RelA-deficient Tregs, since a strong bias was observed for signatures typical of activated Tregs. This reduced capacity of RelA-deficient Tregs to acquire an activation status could be due to an alteration of the proper function of the multimolecular complex normally containing Foxp3, p300, Helios, RelA, and other TFs acting as transcriptional activator (27).

What was more consistent and unexpected was the increased instability of RelA-deficient Tregs. This was first suggested in the colitis model, but more direct evidence came from studies where we compared RelA-sufficient and -deficient Tregs in the same environment after cell co-transfer in lymphopenic mice. We clearly showed that most RelA-deficient Tregs became ex-Tregs, contrary to control Tregs. Although with reduced intensity, increased instability of Rela-deficient Tregs was also observed in the absence of inflammation, as measured after transfer in lymphoreplete mice (data not shown). Moreover, we detected low amounts of the truncated RelA protein in the Tconvs of *Foxp3*^{Cre} *Rela*^{lox} mice, which may reveal the existence of ex-Tregs in these mice. Furthermore, we showed that these newly RelA-deficient ex-Tregs expressed inflammatory cytokines, suggesting that they could become pathogenic. This phenomenon may explain the increased severity observed in the colitis experiment and support our hypothesis that this ex-Tregs contribute to the pathology of *Foxp3*^{Cre} *Rela*^{lox} mice.

Foxp3 stability is controlled by histone and protein acetylation and by DNA methylation in the CNS 2 of *Foxp3* (44). RelA activity may impact on these epigenetic modulations by different ways. RelA interacts with CBP and p300 histone/protein acetyltransferases, which seems to be critical for the recruitment of CBP and p300 to their target promoter sites, as shown in fibroblasts (45). Because CBP and p300 promote *Foxp3* transcription, *Foxp3* stability at the level of CNS2 and prevent Foxp3 degradation (17, 46), RelA-deficient Tregs may have major instability. It has also been recently reported that RelA binds to genes involved in histone modification (29). Also, Foxp3 and RelA seem to cooperate to promote Foxp3 and CD25 expression by binding to their regulatory sequences (47, 48), which may favor Treg stability given the known role of IL-2 receptor signaling pathway in maintenance of Treg identity (16). Furthermore, Oh et al. recently reported that Foxp3 expression was down-regulated in Tregs of *Foxp3*^{Cre} *cRel*^{lox} mice and even more in the *Foxp3*^{Cre} *cRel*^{lox} *RelA*^{lox} mice, suggesting that RelA favors Foxp3 expression (30). Interestingly, we observed a dramatic increased binding of c-Rel to its target DNA sequence in Tregs of *Foxp3*^{Cre} *Rela*^{lox} mice. This phenomenon may hide the genuine role of RelA in Tregs and may further increase their

conversion in pathogenic cells since c-Rel has been reported to be involved in Th1 and Th17 differentiation (36, 37).

Overall, our study further confirms the non-redundant role of RelA in Treg biology and reveals its new role in Treg stability. There are drugs targeting NF-κB subunits. Thus, it would be of strong interest to be able to target RelA in Tregs to propose new therapies triggering or inhibiting Tregs in autoimmune diseases or cancer, respectively. However, RelA has an important role in development and function of other immune cells (49–51). For instance, RelA is critical for $CD4^+$ Tconv activation since its deletion prevent the development of autoimmunity in *Foxp3*^{Cre} *Rela*^{lox}mice (28). Also, RelA is essential for differentiation and function of Th1, Th2, Th17, and Th9 cells (37, 52, 53). Therefore, a specific targeting of RelA in Tregs would be required.

EXPERIMENTAL PROCEDURES

Mice

Foxp3-CRE-IRES-YFP (*Foxp3*^{Cre}) (54), *RelA*^{flox} (31) and *Foxp3-IRES-GFP* (55) knock-in (*Foxp3*^{GFP}) mice were kindly given by Prs. Alexander Rudensky, Falk Weih and Bernard Malissen, respectively. *CD3e*^{tm1Mal} (*CD3*$^{-/-}$), *CD45.1*, *CD90.1*, and *RAG2*$^{-/-}$ mice were obtained from the cryopreservation distribution typing and animal archiving department (Orléans, France). All mice were on a C57Bl/6 background. Mice were housed under specific pathogen-free conditions. All experimental protocols were approved by the local ethics committee "Comité d'éthique en expérimentation animal Charles Darwin N°5" under the number 02811.03 and are in compliance with European Union guidelines.

Western Blot

Cells were lysed for 20 min on ice in extraction buffer (0.4 M NaCl, 25 mM Hepes pH 7.7, 1.5 mM MgCl2, 0.2 mM EDTA, 1%, NP4O, 20 mM glycerol phosphate, 0.2 mM Na3VO4, 10 mM PNPP, 2 mM DTT, 0.1 M PMSF). Whole cell extract was harvested after centrifuging the lysate for 10 min at 9,500 × g. 20 μg of whole cell extract were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare). Immunoblotting was performed with anti-RelA (C20) polyclonal antibodies (Santa Cruz Biotechnology) and anti-β-actin antibody (Sigma Aldrich) and visualized using the ECL Western blotting detection kit (Pierce).

Histology

Organs were collected and fixed in PBS containing 4% formaldehyde for 48 h and then transferred in 70% ethanol. Five-micrometer paraffin-embedded sections were cut and stained with hematoxylin and eosin and then blindly analyzed.

Cell Preparation From Tissues

For lymphoid tissues, cells were isolated by mechanical dilacerations. For non-lymphoid tissues, anesthetized mice were perfused intracardially with cold PBS. Small pieces of livers and lungs were digested in type IV collagenase (0.3 mg/ml) and DNase I (100 μg/ml) for 30 min at 37°C, followed by Percoll gradient (30–70%) separation. Small pieces of intestines, removed of their Peyer patches and epithelium, were digested in type IV collagenase (1 mg/ml) and DNase I (10 μg/ml) for 30 min at 37°C, followed by Percoll gradient (40–80%) separation. Small pieces of skin were digested in liberase DL (0.4 mg/ml), collagenase D (0.05 mg/ml) and DNase I (10 μg/ml) for 1 h at 37°C, followed by Percoll gradient (40–80%) separation.

Antibodies and Flow Cytometry Analysis

The following mAbs from BD Biosciences were used: anti-CD45 (30-F11), anti-CD8 (53-6.7), anti-CD4 (RM4-5), anti-CD62L (MEL-14), anti-CD90.1 (OX-7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD25 (PC61 or 7D4), anti-ICOS (7E.17G9), anti-GITR (DTA-1), anti-CD103 (M290), anti-Helios (22F6), anti-CTLA-4 (UC10-4F10-11), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD19 (1D3), anti-IA/E (M5/114.15.2), anti-Ly6C (AL-21), anti-Ly6G (1A8). Anti-GFP antibody was purchased from Life Technologies. Anti-CD3 (145-2C11), anti-Foxp3 (FJK-16s), anti-CD44 (IM7), anti-Ki-67 (SOLA15), anti-Nrp1 (3DS304M), anti-NKp46 (29A1.4) and anti-F4/80 (BM8) were purchased from eBioscience, and Foxp3 staining was performed using the eBioscience kit and protocol. Cells were acquired on a BD LSRII and a BD Fortessa X20 cytometers and analyzed using FlowJo software.

Cytokine Quantification

Serum cytokines were quantified using the mouse Th1/Th2/Th17 Cytokine CBA Kit (BD Biosciences) according to manufacturer's procedure. Datas were analyzed using FCAP array software.

Immunoglobulin and Autoantibody Quantification by ELISA

Ninety-six-well flat plates were coated with either salmon sperm DNA (Sigma) or with goat anti-mouse IgM, IgA, IgE, IgG1, IgG2b (Southern Biotech). After washes, they were saturated with BSA and first incubated with mice sera, then with biotinylated goat anti-mouse IgG (Southern Biotech) or goat anti-mouse IgM, IgA, IgE, IgG1, IgG2b (Southern Biotech). A streptavidin-horseradish conjugate (Sigma) was added followed by the addition of TMB (eBioscience). The reaction was stopped with HCl (1N) and revealed with an ELISA plate reader DTX880 Multimode Detector (Beckman Coulter).

Treg and Tconv Cell Purification

Treg were purified after enrichment of $CD25^+$ cells using biotinylated anti-CD25 mAb (7D4) and anti-biotin microbeads (Miltenyi Biotec), followed by CD4 staining (RM4.5) and cell sorting of $CD4^+$ $Foxp3/YFP^+$ cells or CD4+ $Foxp3/GFP^+$ using the BD FACSAria II. Tconv cells were purified after enrichment of $CD25^-$ cells using biotinylated anti-CD25 mAb (7D4) or of $CD8^-CD19^-CD11b^-$ cells using biotinylated anti-CD8 (53-6.7), CD19 (1D3), and CD11b (M1/70) mAbs and anti-biotin microbeads (Miltenyi Biotec), followed by CD4 staining (RM4.5), and cell sorting of $CD4^+$ $Foxp3/YFP^-$ cells or CD4+ $Foxp3/GFP^-$ using the BD FACSAria II.

Cell Cultures

Purified Treg ($CD4^+YFP^+$, 25×10^3 cells/well) were cultured with or without whole splenocyte from CD3KO mice (7.5×10^4 cells/well), anti-CD3 mAb (0,05 µg/ml, BioXcell), TNF (50 ng/ml, Protein Service Facility, VIB, Belgium) and IL-2 (10 ng/ml, Peproteck) in a 96-well round plate in RPMI 1640–10% FCS. For suppression assays, after labeling with CellTrace Violet Proliferation Kit (Life technologies), Tconv cells ($CD4^+YFP^-$, 2.5×10^4 cells/well) were co-cultures with various Treg ($CD4^+YFP^+$) numbers and stimulated by splenocytes from CD3 KO mice (7.5×10^4 cells/well) and soluble anti-CD3 (0.05 µg/ml 2C11, BioXCell) in RPMI 1640–10% FCS.

Colitis

Tconv cells ($CD4^+GFP^-$, 1×10^5 cells) and Tregs ($CD4^+YFP^+$, 2×10^4 cells) were injected intravenously into sex-matched $RAG2^{-/-}$ mice. The clinical evaluation was performed three times a week by measuring body weight. Colitis was scored on tissue sections as described previously (56).

T-Cell Adoptive Transfer

CD3 KO mice were co-transferred with Treg ($CD4^+YFP^+$, 1×10^5 each) purified from age and sex-matched CD45.1/2 $Foxp3^{Cre/+}$ and CD45.2/2 $Foxp3^{Cre/+}$ $Rela^{lox}$ mice and Tconv cells ($CD4^+GFP^-$, 8×10^5) purified from CD90.1 $Foxp3^{GFP}$ mice.

Electrophoretic Mobility Shift Assays (EMSA) Combined With Supershit Assays

Nuclear extracts were prepared and analyzed for DNA binding activity using the HIV-LTR tandem κB oligonucleotide as κB probe (57). For supershift assays, nuclear extracts were incubated with specific antibodies for 30 min on ice before incubation with the labeled probe.

Gene-Expression Profiling and Analysis

Tregs (1,000) were double-sorted into TRIzol (Invitrogen). Subsequent sample processing was followed by Ultra-low input RNAseq protocol as described (58). Normalized data were analyzed with Multiplot Studio, GSEA and Gene-e modules in Genepattern. For signature enrichment analysis, each signature was curated from published datasets and computed by comparison between two conditions (e.g., WT vs. KO). Data were downloaded from GEO and only the ones containing replicates were used. To reduce noise, genes with a coefficient of variation between biological replicates > 0.6 in either comparison groups were selected. Up- and down-regulated transcripts were defined as having a fold change in gene expression > 1.5 or $< 2/3$ and a t-test p-value < 0.05. A signature score for each single cell was computed by summing the counts for the upregulated genes and subtracting the counts for the downregulated genes. Z scores were plotted in the heat map (Zemmour_Code/Zemmour_Code.Rmd: **Treg signatures and single cell score**).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism Software. Statistical significance was determined using a log-rank (Mantel- Cox) test for the mouse survival data. For all the other statistical analysis, the two-tailed unpaired non-parametric Mann–Whitney U-test was used for data not following a normal distribution and the t-test was used for data following a normal distribution. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Means ± SEM were used throughout the figures.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All experimental protocols were approved by the local ethics committee Comité d'éthique en expérimentation animal Charles Darwin N°5 under the number 02811.03 and are in compliance with European Union guidelines.

AUTHOR CONTRIBUTIONS

BS and ER designed the research. ER performed almost all the experiments and analyzed the data. ML, RV, JD, SG, and AR helped ER on some experiments. H-KK and CB performed and analyzed the RNA-seq data. DC and VB performed and analyzed the western blot and EMSA. BS and ER wrote the manuscript using comments from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02487/full#supplementary-material

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REVIEW
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Stability and Maintenance of Foxp3$^+$ Treg Cells in Non-lymphoid Microenvironments

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Foxp3$^+$ Treg cells are indispensable for maintaining self-tolerance in secondary lymphoid organs (SLOs). However, Treg cells are also recruited to non-lymphoid tissues (NLTs) during inflammation. Recent advances in the understanding of Treg cell biology provided us with molecular mechanisms—both transcriptional and epigenetic—that enable Treg cells to retain their identity in an inflammatory milieu that is *per se* hostile to sustained expression of high levels of Foxp3. While Treg cells are recruited to sites of inflammation in order to resolve inflammation and re-establish appropriate organ function, it is increasingly recognized that a series of inflammatory (but also non-inflammatory) perturbations of organ function lead to the constitution of relatively long lived populations of Treg cells in NLTs. NLT Treg cells are heterogeneous according to their respective site of residence and it will be an important goal of future investigations to determine how these NLT Treg cells are maintained, e.g., what the role of antigen recognition by NLT Treg cells is and which growth factors are responsible for their self-renewal in the relative deficiency of IL-2. Finally, it is an open question what functions NLT Treg cells have besides their role in maintaining immunologic tolerance. In this review, we will highlight and summarize major ideas on the biology of NLT Treg cells (in the central nervous system but also at other peripheral sites) during inflammation and in steady state.

Keywords: Treg—regulatory T cell, Foxp3, heterogeneity, central nervous system, stability, non-lymphoid tissues

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INTRODUCTION

Foxp3$^+$ Treg cells have been intensely studied over the last three decades (1). Treg cells in secondary lymphoid tissues are indispensable for maintaining immune tolerance because elimination of Treg cells either in newborn individuals or in adults results in multiorgan autoimmunity within a couple of weeks (2, 3). It is an intriguing concept to exploit Treg cells for therapeutic interventions—either enhancing their function in autoimmunity or dampening their effect in cancer. Proof of concept trials in graft-vs.-host disease and even in type 1 diabetes have been undertaken in humans (4, 5). However, antigen specificity of Treg cells (most trials were performed with polyclonal Treg cell populations), their trafficking behavior, and their stability after adoptive transfer into human hosts remain challenges on the way to a broader application of adoptive Treg cell therapy (6). Perhaps it is now time to take a step back and consider novel and unconventional concepts about Foxp3$^+$ Treg cells that might nevertheless be fundamental to the understanding of Treg cells in homeostasis and in disease conditions: First, Treg cells might have some plasticity and adapt to the functional

context, in which they need to be operational (7). For instance, during inflammation, Foxp3$^+$ Treg cells upregulate pathways that secure the preservation of their identity as Treg cells but might have additional (as yet unknown) functions for the establishment of tissue-resident Treg cell subsets. Second, Treg cells do not only reside in secondary lymphoid tissue but also in NLT. These "tissue-resident" Treg cells are distinct from circulating lymphoid tissue Treg cells and might in some cases populate distinct tissue niches (8). Limited information exists as to whether tissue-resident Treg cells are differentially recruited from the systemic repertoire or whether their functions are imprinted *in situ* in their particular niche. Also, their TCR repertoire and the role of antigen for their maintenance is not known. Finally, they might exert "non-canonical" functions in these tissues that do not have anything to do with the regulation of immune responses in the first place but with tissue development and organ homeostasis. In this review, we will discuss some of these aspects in the central nervous system (CNS) and in those peripheral organs where Treg cells have been investigated in non-lymphoid tissue niches.

STABILITY OF FOXP3 TREG CELLS IN THE CNS IN THE CONTEXT OF AUTOIMMUNITY

Treg cells are crucial for the regulation of autoimmune inflammation in the CNS. Depletion of Treg cells lowers the threshold for autoimmune CNS inflammation in individuals whose T cell receptor repertoire contains large fractions of CNS reactive T cells (9). Moreover, depletion of Treg cells prior to or after onset of experimental autoimmune encephalomyelitis (EAE) worsens the disease and prevents recovery (10–12).

Since it is clear that Foxp3$^+$ Treg cells are recruited to the target tissue of autoimmune reactions not only in the CNS (13, 14) but also in other organs including the joints (15), the pancreas (16), or the skin (17, 18), a major area of investigation in Treg cell biology in the recent years has been their stability in an inflammatory environment. Since it has been recognized that Foxp3$^+$ Treg cells are recruited directly to the site of inflammation, Treg cells must dispose of active mechanisms of resilience to maintain their functional phenotype in spite of inflammatory cues in their environment. A variety of pathways have been described, which all ultimately result in keeping the expression of Foxp3 at high levels when factors of the inflammatory milieu activate pathways that otherwise would destabilize Foxp3 expression. The overarching concept is that Foxp3 interacts with (16–19) or is co-expressed with various combinations of transcription factors in Treg cells to induce an effector Treg (eTreg) program and to adapt to the quality of the inflammatory response that is supposed to be controlled by these Treg cells (19–21) while at the same time preserving their identity as Treg cells. Here, direct transactivators of Foxp3 as well as transcriptional inhibitors of effector T cell programs have been described (**Table 1**).

Moreover, the significance of epigenetic modifications both of the chromatin in the vicinity of the Foxp3 locus and of the Foxp3 locus itself in regulating the expression of Foxp3 in distinct milieus is increasingly appreciated (27, 28). In addition to the promoter of Foxp3, three conserved non-coding regions (*CNS1-3*) have been identified in the Foxp3 locus, whose methylation status determines the efficacy with which *Foxp3* is transcribed since for instance, Ets-1 transcription factors only bind to *CNS2* [i.e., the conserved non-coding sequence in the first intron of the *Foxp3* locus that has also been termed Treg specific demethylated region (TSDR) (29)] in its demethylated state and thus increase the enhancer activity of *CNS2* for *Foxp3* (30).

During local inflammation, the central nervous system milieu represents a particular challenge to the identity and function of eTreg cells. The most relevant molecular mechanisms that preserve the "identity" of Treg cells (e.g., their sustained expression of Foxp3) have been a matter of debate. Recently, it has been shown that both TCR/Irf4 signaling and NFκB signaling are required independently of each other to establish the eTreg cell transcriptional program (31, 32); and the transcriptional modifier Blimp1 is a master controller of the eTreg program in Treg cells (33). Loss of Blimp1 in Treg cells in steady state does not produce an inflammatory phenotype, most likely due to the fact that steady state Treg cells in secondary lymphoid tissue only express low levels of Blimp1. In contrast, loss of Blimp1 in Treg cells in the inflamed CNS or in the colon has major consequences: First, these NLT Treg cells lose their effector Treg phenotype. For instance, since Blimp1 is a direct transactivator of *Il10*, Blimp1 deficient Treg cells are unable to produce IL-10 (34–36). Second, Blimp1 is also required to maintain the very identity of Treg cells in an inflammatory milieu and loss of Blimp1 eventually leads to downregulation of Foxp3 expression (35). Mechanistically, *Foxp3* is not a transcriptional target of Blimp1. Rather Blimp1 appears to be controlling the expression of Foxp3 in an indirect manner by preventing the inflammatory environment (and particularly IL-6) from methylating *CNS2*. In fact, Blimp1 suppresses the expression of the methyl transferase Dnmt3a in Treg cells in an inflammatory environment (35).

In principle, genomic CpG islands are methylated and demethylated by DNA methyltransferases and Tet methylcytosine dioxygenases, respectively (37). The modulation of the demethylating enzymes Tet2 and Tet3 in Treg cells affects *CNS2* methylation and Treg cell stability (38). Notably, the activity of the Tet enzymes might be controlled by intermediates of the mitochondrial tricarboxylic acid cycle. In fact, ablation of the mitochondrial transcription factor A (Tfam) leads to altered succinate/α-ketoglutarate and fumarate/α-ketoglutarate levels in Treg cells—and as a consequence—to reduced activity of the Tet enzymes and a failure to maintain the demethylated state of *CNS2* (39). By this mechanism, intermediary metabolism in Treg cells is coupled to their stability. Conversely, DNA methyltransferases (and in particular Dnmt1 and Dnmt3a) are able to methylate *CNS2* (and other CpG islands in the *Foxp3* locus) and thus dampen Foxp3 expression. Here, an interesting model has recently been suggested (40): STAT5 (downstream of IL-2) binds to *CNS2* irrespectively of the methylation status of *CNS2* and increases its enhancer activity as to the expression of Foxp3 (41). Both STAT3 (downstream of IL-6) and STAT6 (downstream of IL-4) can compete with STAT5 for its binding sites in *CNS2*.

TABLE 1 | Selection of molecules directly involved in the transcriptional regulation of Foxp3 in murine NLT Treg cells.

Modulator	Mechanism	References
Runx-CBFβ	Occupation of *Foxp3* promoter and CNS2. Also relevant for steady-state Foxp3 expression.	(22)
Foxp1	Foxp1 co-occupies Foxp3 target loci. Negative regulation of Satb1 expression in Treg cells.	(23)
HIF1α	Exaggerated expression of HIF1α in Treg cells (by ablation of the E3 ubiquitin ligase VHL) leads to their metabolic reprogramming into effector T cells.	(24)
DBC1	DBC1 physically interacts with Foxp3 and renders the complex more susceptible to inflammation induced degradation.	(25)
Pak2	Treg cells deficient in p21-activated kinase 2 (Pak2) convert into Th2 cells with high Gata3 expression.	(26)

In contrast to STAT5, both STAT3 and (more prominently) STAT6 appear to physically interact with Dnmt1 and Dnmt3a (40, 42), and thus can mediate their recruitment to *CNS2*. Methylation of *CNS2* by these methyl transferases would then lead to the silencing of *Foxp3*. This model provides a rationale for inflammation (IL-4 and IL-6) induced downregulation of Foxp3 in Treg cells and IL-2 dependent counteraction of this process.

Apart from epigenetic modulation of the *Foxp3* locus itself, histone modifications also control Foxp3 transcription. Interestingly, partial methylation of *CNS2*, which occurs in Treg cells exposed to an inflammatory environment, results in the recruitment of methyl CpG binding protein 2 (MeCP2) to the methylated CpG islands of *CNS2*. MeCP2 in turn leads to the acetylation of H3, which reinforces Foxp3 transcription (43). Also, Ezh2, a chromatin modifying enzyme that catalyzes the trimethylation of H3K27, is co-expressed in Treg cells in response to CD28 stimulation. Foxp3 cooperates with Ezh2 to repress its target genes, and in the absence of Ezh2, Foxp3 is still present in Treg cells but fails to reinforce its transcriptional program (44).

Taken together, the stability of Foxp3$^+$ Tregs at sites of inflammation is instructed by an active process. Treg cell extrinsic cues (TCR stimulation, co-stimulation, cytokines) are required to secure the stability of Treg cells and both direct transcriptional as well as epigenetic mechanisms are involved in maintaining high levels of Foxp3 expression and thus the functional phenotype of Treg cells in inflamed tissues.

MAINTENANCE OF NLT FOXP3$^+$ TREG CELLS DURING AND AFTER INFLAMMATION

Compelling evidence suggests that IL-2 is a non-redundant growth factor of Treg cells in secondary lymphoid tissues. Treg cells do not produce IL-2 themselves but rely on extrinsic sources of IL-2, which is mostly provided by conventional T cells. Lack of IL-2 leads to the attrition of Treg cells and to the development of multi-organ autoimmunity (45). Mechanistically, IL-2 counteracts the apoptosis prone transcriptional program of Treg cells (46). It is quite likely that during organ specific inflammation—due to massive infiltration of conventional T cells—the source of IL-2 is sufficient also in NLTs to fuel the maintenance and even expansion of Foxp3$^+$ Treg cells (47). In fact, a decrease in the threshold of IL-2 responsiveness in Treg cells or more efficient STAT5 signaling, which can be modulated by a plethora of mechanisms (23, 40, 48), have been identified as a key "stability" mechanism of Treg cells in NLTs under conditions of limited availability of IL-2 (see above).

However, after the contraction of the effector T cell population in the target tissue of the inflammation, the source of IL-2 (and thus the relevant growth factor for Treg cells) becomes limiting. As a consequence, the Treg cell population in NLTs will also contract (47). However, we and others have observed that after a traumatic or inflammatory event in the central nervous system (but also in other tissues), Foxp3$^+$ Treg cells remain in the central nervous system for extended periods of time and might even establish a population of resident Treg cells in the relative absence of conventional T cells (13). Little is known about this "meta-homeostatic" Treg cell population (**Figure 1**). Previous studies suggested that Treg cells which infiltrate the CNS during EAE were most likely exclusively thymus-derived and not peripherally induced from conventional T cells (13). In particular, as mice, which are unable to generate peripherally induced Treg cells due to a mutation of the *Foxp3* locus lacking *CNS1* but have regular numbers of thymus derived Treg cells, do not have an EAE phenotype, Treg cells in the inflamed central nervous system must be thymus-derived (49). However, it is an open question how this NLT Treg cell population is selected or whether its specific properties are locally imprinted and also how it is maintained.

In skin and colon NLT Treg cells have been characterized by scRNAseq as to a specific "barrier tissue" transcriptional program and as to their provenance and development (in pseudotime analyses) (50). By analyzing NLT Treg cells, it has been possible to define a transcriptional program reminiscent of the effector Treg (eTreg) phenotype that has been coined for Treg cells isolated from the visceral adipose tissue (33, 51). While definitive proof by provenance mapping systems is still lacking, based on these scRNAseq analysis, the instruction of a Treg cell trait consistent with tissue residency might be initiated in secondary lymphoid tissues. Further imprinting of functional states then occurs in the NLT (**Figure 2**). Best evidence for this model has been presented for VAT Treg cells: By using PPAR-γ reporter mice, a previous study has identified a small population of PPAR-γ^{low} expressing Treg cells in secondary lymphoid organs (SLO), in which part of the VAT Treg transcriptional program, in particular those genes associated with Treg activation, was

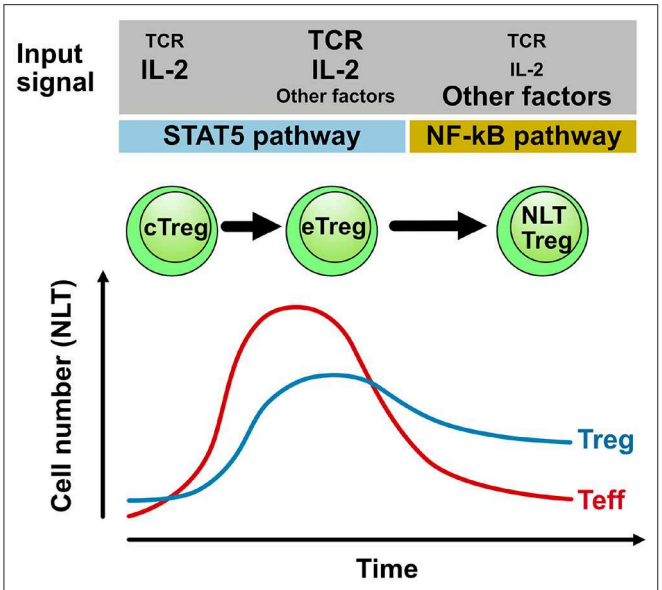

FIGURE 1 | Population dynamics and maintenance of Treg cells in NLTs. During an adaptive immune response in NLT (e.g., in the central nervous system during EAE) conventional T cells (Teff) infiltrate the target tissue and expand providing sufficient IL-2 to also drive the differentiation and expansion of Treg cells. After contraction of the conventional effector T cell (Teff) population (due to active regulation by Treg cells), IL-2 becomes limiting and persisting Treg cells likely depend on alternative signals for self-renewal. Here, signals that activate the NF-κB pathway might be important cues not only for NLT function but also for their maintenance (31). TCR triggering of Treg cells and downstream modulation of the TCR signal by the transcriptional modulator IRF4 is a non-redundant event in the differentiation of central Treg cells (cTreg) in secondary lymphoid tissues into effector Treg cells (eTreg) in the inflamed tissue. The requirement of the TCR signal and other cues in NLT Treg cells that reside in specific niches over extended periods of time needs to be determined and likely depends on the anatomical niche that might then also drive a distinct functional specialization of NLT Treg cells.

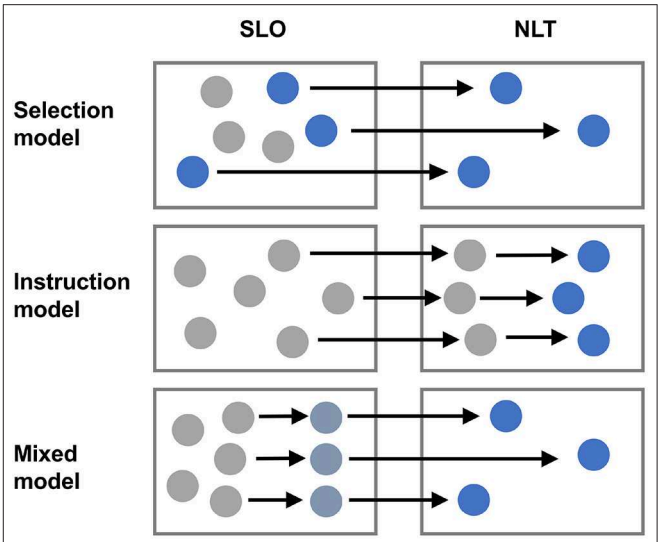

FIGURE 2 | Heterogeneity of Treg cells in NLTs. Treg cells are heterogeneous and the NLT milieu is likely an important determinant of this heterogeneity. However, it is unclear whether specific hard-wired subsets of Treg cells are selectively recruited from SLOs to distinct NLTs (selection model) or whether Treg cells that enter a given NLT are locally instructed to adopt specific properties and specialize according to a niche specific program (instruction model). Alternatively, some degree of imprinting might occur in SLOs during priming and further commitment of Treg cells to certain tissue specific properties might then take place in NLTs (mixed model).

already active, and these cells were shown to be capable of adopting the full VAT Treg signature upon their migration into the tissue (52), most likely in response to local tissue-specific cues. Based on this finding, it was proposed that the bona-fida VAT Treg gene signature/phenotype might be adopted in a sequential, step-wise process, that is initiated in the SLO and then finalized within the non-lymphoid compartment. The first step appears to be a "priming" step in SLOs, where Treg cells receive an activating cue, e.g., in response to cognate self antigen recognition. Then they gain the capacity to infiltrate into NLTs. In a second step, Treg cells that re-encounter their specific antigen in the NLT, might be preferentially retained *in situ* and receive further tissue-specific cues that impart the full NLT phenotype and function (52).

In particular, NFκB (RelA) signaling appears to be a hallmark of the eTreg cell [and NLT Treg cell) program. Whether TNF family members (including TNF-ligand related molecule 1 (31, 53)] or IL-1 family members [including IL-33 (51) and IL-18 (54)] are more crucial or differentially important to guide the NLT Treg program, remains to be determined. Also, it remains to be determined whether NFκB signaling into Treg cells secures their maintenance outside SLOs where IL-2 derived from conventional T cells, which is a major nutrient of Treg cells in lymphoid tissues, is scarce.

Very little is known about the "Treg cell growth and maintenance" factors in NLTs and whether they are universal for all peripheral tissues or private to distinct anatomical niches. The alarmin IL-33, a member of the IL-1 family of cytokines, plays a major role in driving the expansion/accumulation of VAT-resident Treg cells (51, 52, 55). IL-33 is constitutively expressed in the CNS even under physiological conditions (56). Given the high expression of its receptor ST2 (encoded by *Il1rl1*) on brain Treg cells and the observation that Treg cells fail to properly expand in the injured ischaemic brain in ST2- and IL-33-deficient mice (57), IL-33 might be a prominent candidate capable of substituting for the role IL-2 in mediating Treg cell survival in the post-inflammatory central nervous system. Another such candidate could be the neurotransmitter serotonin (5-HT). Its receptor 5-HT7 protein (encoded by *Htr7*) is specifically upregulated on Treg cells accumulating in the ischaemic brain (57), and it was demonstrated that both serotonin or the inhibition of its uptake could expand brain Treg cell numbers *in vivo*.

In summary, NLT Treg cells (in the central nervous system but also in other tissues) likely populate niches that provide a distinct environment for their survival. The molecular pathways that support their survival (and possible self renewal) are likely different from the IL-2 driven Treg cell maintenance system in secondary lymphoid organs.

NON-CANONICAL FUNCTIONS OF TREG CELLS

While thymus-derived Treg cells that populate SLOs are non-redundant in maintaining self tolerance, eTreg cells in NLTs also clearly regulate effector responses of conventional T cells during adaptive immune responses in order to clear inflammation and reduce immune pathology. However, since Treg cells are found in NLTs in steady state and after re-establishment of homeostasis after any kind of injury (meta-homeostasis), it has been suggested that tissue intrinsic (non-immune) functions of these peripheral tissues might be controlled by local Treg cells (8).

Various NLTs, including barrier tissues like the skin, the colonic lamina propria and the lung, as well as non-barrier sites, like the visceral adipose tissue (VAT) of lean mice or the skeletal muscle are populated by a distinct population of regulatory T cells. As outlined above, NLT Treg cells show a high degree of phenotypic and functional adaptation to the NLT environment in which they reside and share a set of common NLT-specific transcripts that distinguish them from their counterparts in SLOs. The vast majority of NLT Treg cells display an elevated expression of genes typically associated with an eTreg phenotype, such as the effector molecules Ctla4, Icos, Klrg1, and GzmB. Despite a certain overlap in the transcriptomes between NLT Treg cells from different NLTs, there are a number of transcripts specifically and distinctively upregulated in Treg cells from particular non-lymphoid compartments, which might endow these NLT Treg cells with specialized functions in organ homeostasis.

For example, VAT Treg cells highly express the transcription factor peroxisome proliferator-activated receptor (PPAR)-γ, the master regulator of adipocyte differentiation. PPAR-γ governs much of the unique transcriptional signature of VAT Treg cells (58), including the expression of genes involved in lipid metabolism, such as *Dgat1* (diacylglycerol O-acyltransferase 1), encoding an enzyme involved in triacylglycerol biosynthesis, *Pcyt1a* (choline-phosphate cytidylyl transferase A), which encodes an enzyme involved in phosphatidylcholine synthesis, as well as *Cd36*, encoding the lipid scavenger receptor CD36 (59). Specific deletion of PPAR-γ in Treg cells (using Foxp3-Cre x $Pparg^{flox/flox}$ mice) impairs their accumulation in VAT, but not in SLOs, and promotes an increased inflammatory state in the VAT associated with enhanced systemic insulin resistance (59).

In other NLTs, further functions of resident Treg cells have been described that are unrelated to the control of immune responses in the first place. For example, in the steady state skin, $Foxp3^+$ Treg cells are localized in close proximity to hair follicles where they promote the proliferation of hair follicle stem cells and the telogen to anagen transition of the hair follicle by providing the Notch-ligand Jag1, which is sensed by hair follicle stem cells (60). In lung tissue and in muscle tissue, Treg cells contribute to tissue regeneration after injury by producing the epidermal growth factor receptor ligand amphiregulin, to which bronchial epithelial cells respond with proliferation and differentiation (54) and muscle satellite cells respond with myogenic differentiation (61), respectively.

In contrast to NLT such as the VAT, intestine, lung or skin, "immune-privileged" compartments like the central nervous system are largely devoid of Treg cells during steady state conditions. However, Treg cells readily accumulate in the CNS in response to acute local injury (e.g., hypoxia/stroke) (62) or autoimmune inflammation (13). Interestingly, Treg cell numbers remain elevated in the post-EAE central nervous system for a prolonged period of time, raising the question of whether these cells might also play a significant role in promoting tissue repair in the recovering central nervous system. In fact, Treg cells have already been shown to be capable of supporting myelin regeneration by promoting the differentiation of oligodendrocyte progenitor cells and their production of myelin in both the brain and the spinal cord *in vivo* in a model of toxic demyelination (lysolecithin or cuprizone injection) (63). Central nervous system Treg cells have a transcriptional profile that clearly differs from that of SLO Treg cells (35, 57), and in many ways resembles the profile of other NLT Treg populations. Microarray analysis of Treg cells that had infiltrated the brain after acute ischaemic injury revealed that these Treg cells appear to be transcriptionally related to VAT and skeletal muscle Treg cells, as demonstrated by the high level expression of genes that encode IL-10, amphiregulin (*Areg*); Klrg1, ST2, and PPAR-γ. In addition, brain Treg cells upregulate certain central nervous system-specific genes, such as neuropeptide Y (*Npy*), preproenkephalin (*Penk*), serotonin receptor type 7 (*Htr7*), and arginine vasopressin receptor (*Avpr1a*) (57). In particular the increased expression of the epidermal growth factor ligand amphiregulin by CNS recruited Treg cells seems important for neurological recovery as demonstrated by its capacity to suppress astrogliosis, neurological deficits and neurotoxic gene expression (57).

Therefore, NLT Treg cells might be private to their own niche of residence and intricately connected to tissue intrinsic non-immune processes in homeostasis and organ development.

HUMAN NLT TREG CELLS

NLT Treg cells have been extensively studied in mice, however it has become clear that essentially all human NLTs also harbor a distinct population of Treg cells (64, 65). The functional phenotype of human NLT Treg cells remains largely unexplored and it is possible that pathways identified as relevant for the maintenance and function of murine NLT Treg cells may not have the same importance for human NLT Treg cells. For example, omental fat tissue—the human correlate of murine VAT—has been reported to contain detectable levels of Foxp3 transcripts (58). Like their counterparts in mice, omental fat Treg cells have been shown to express ST2 (51) while other studies failed to detect ST2 expression on $Foxp3^+$ Treg cells isolated from human omental adipose tissue and also other organs including colon and lung (66, 67), suggesting that the IL-33/ST2 axis may not be a universal pathway for the maintenance of human NLT Treg cells.

Treg cells are also abundant in healthy adult human skin, comprising about 20% of the total $CD4^+$ T cell population.

Human skin Treg cells exhibit an activated memory phenotype, as defined by their almost uniform expression of the memory markers CD45RO, CD27, and Bcl-2 and the increased expression of Foxp3 and Treg activation markers, such as CTLA-4, CD25, and ICOS relative to their counterparts in the peripheral blood. There is little overlap in the TCR repertoire between Treg cells and conventional T cells in healthy human skin, suggesting that these two NLT T cell populations recognize different antigens, similar to what has been observed for Treg cells and conventional T cells from murine VAT (68). Interestingly, similar to their murine equivalents (60), dermal Treg cells in normal human skin were found to preferentially reside in close proximity to hair follicles (68).

The currently available data suggest a considerable overlap in the expression of tissue-specific Treg cell markers between mice and humans, as demonstrated for skin- or gut-derived Treg cells (50). Also, the NLT core signature which is characterized by an enrichment of genes related to the TNFRSF-NF-κB pathway appears to be largely conserved between mouse and human Treg cells (50, 69), raising the possibility that human NLT Treg cells might undergo a similar process of local imprinting and tissue adaptation during their establishment in NLTs.

PERSPECTIVE

Fundamental questions regarding NLT Treg cells are as yet unresolved: For example, during organ specific inflammatory responses, Treg cells that are recruited to the sites of inflammation are faced with issues of stability of their phenotype and deficiency in growth factors that they would otherwise be able to rely on in steady state in SLOs. Also, it is unclear whether subsets of Treg cells are pre-committed to certain phenotypes in the secondary lymphoid system and are then selectively recruited to certain anatomical niches where they expand or whether "naive" Treg cells are locally instructed to adopt a distinct phenotype only after the recruitment to specific organ systems. Answers to these questions will profoundly affect concepts of therapeutic interventions that intend to make use of tissue-resident Treg cells. It might in fact be oversimplified (and perhaps even dangerous) to aim for a "one-size-fits-all" Treg cell. Rather, more tailored strategies to either selectively expand subsets of Treg cells or to instruct them to adopt appropriate (organ-specific) features might have to be developed. Here, we face dramatic gaps in knowledge and a renewed interest in Treg cell centered therapeutic approaches that exploit the immune functions of Treg cells needs to consider the emerging profound organ specific heterogeneity of $Foxp3^+$ Treg cells and their potential functions in tissue homeostasis and regeneration.

AUTHOR CONTRIBUTIONS

TK conceptualized and wrote the review. AM wrote parts of the review and edited the text.

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REVIEW
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T-Follicular Regulatory Cells: Potential Therapeutic Targets in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is an incurable aggressive chronic inflammatory joint disease with a worldwide prevalence. High levels of autoantibodies and chronic inflammation may be involved in the pathology. Notably, T follicular regulatory (Tfr) cells are critical mediators of T follicular helper (Tfh) cell generation and antibody production in the germinal center (GC) reaction. Changes in the number and function of Tfr cells may lead to dysregulation of the GC reaction and the production of aberrant autoantibodies. Regulation of the function and number of Tfr cells could be an effective strategy for precisely controlling antibody production, reestablishing immune homeostasis, and thereby improving the outcome of RA. This review summarizes advances in our understanding of the biology and functions of Tfr cells. The involvement of Tfr cells and other immune cell subsets in RA is also discussed. Furthermore, we highlight the potential therapeutic targets related to Tfr cells and restoring the Tfr/Tfh balance via cytokines, microRNAs, the mammalian target of rapamycin (mTOR) signaling pathway, and the gut microbiota, which will facilitate further research on RA and other immune-mediated diseases.

Keywords: T follicular regulatory cell, rheumatoid arthritis, therapeutic targets, T follicular helper cell, germinal centers, immune regulation

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INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease involving damage to the joint synovium and irreversible disability (1). Affecting about five per 1,000 adults, RA threatens the normal work and daily life of patients (2). However, the pathophysiological mechanism of RA is only partly understood. Genotype, environmental factors, and epigenetic modification are implicated in the pathological process of RA (3). Normally, our immune system has extraordinary ability to identify and attack pathogenic microorganisms without targeting self-tissues (4). Genetic defects and environmental factors can lead to deficient immune tolerance, which prevents elimination of autoreactive lymphocytes. This in turn results in abnormal T-cell signaling and autoantibodies, which causes multiple organ inflammation and damage (5). RA is characterized by dysregulated chronic inflammation of the synovial membrane (2, 6). In this inflammatory process, immune homeostasis is disturbed and various abnormal autoantibodies, such as anti-rheumatoid factor (RF) and anti-cyclic citrullinated peptides (anti-CCP), are generated, leading to immune-complex deposition with subsequent destruction of articular cartilage and bone (7, 8).

The generation of autoantibodies related to RA depends on lymphoid follicular germinal centers (GCs). GCs are aggregates of rapidly dividing B cells. In GCs, B cells undergo a series of reactions including affinity maturation, class switch recombination (CSR), and somatic hypermutation, resulting in the generation of large quantities of high-affinity antibodies and memory B cells (9). In this process, T follicular helper (Tfh) cells migrate to GCs and provide the necessary survival, proliferation, and selection signals to cognate B cells (10). Tfh cells are usually characterized as a $CD4^+CXCR5^{hi}PD1^{hi}BCL6^+ICOS^{hi}$ subset in human secondary lymphoid organs (11). The dysregulation of Tfh cells can lead to the production of autoantibodies by B cells. Indeed, the GC reaction requires the regulation and participation of many other cell types, such as monocytes, macrophages, dendritic cells, and neutrophils (12, 13). However, the molecular and cellular mechanisms of autoantibody production are unclear. Traditional therapeutic schedules of RA are based on suppressing excessive immunological responses, which can only achieve stable remission and slow the course rather than cure (12). Moreover, these strategies trigger multiple systemic side effects (2). Therefore, the exploration of novel cellular and/or molecular targets that enable precise regulation of the production of autoantibodies is currently a hot topic.

T follicular regulatory (Tfr) cells, a subpopulation of regulatory T cells (Tregs), have potential for immune regulation within GCs, inhibiting the GC reaction and interacting with Tfh and/or B cells to suppress production of high-affinity antibodies (14–16). Tfr cells are distinguished from other CD4 T-cell subsets predominantly by their $Foxp3^+$ and CXCR5 expression (14–16). Dysregulation of Tfr cells leads to an aberrant GC reaction (14, 16), which contributes to the accumulation of autoantibodies and eventually promotes the development of autoimmune diseases (17). Indeed, the Tfr/Tfh ratio and their function are more important determinants of the B-cell response than the absolute number of Tfr or Tfh cells (18). The balance of Tfh and Tfr cells is disrupted in the peripheral blood of patients with autoimmune diseases, such as RA, systemic lupus erythematosus (SLE), myasthenia gravis (MG), and multiple sclerosis (MS) (19–21). The clinical potential of Tfr cells was confirmed by the finding that the frequency of Tfr cells is negatively correlated with the disease activity score in 28 joints based on C-reactive protein (DAS28-CRP) (22, 23). Furthermore, experimental models have highlighted the therapeutic benefit of Tfr cells in mice with arthritis (24). The targeting of regulatory factors and pathways involved in Tfr differentiation and/or function may be a good therapeutic strategy for RA as well as other autoimmune diseases.

BIOLOGY OF TFR CELLS

Tfr cells were first described as a subset of Tregs with the surface phenotype of $CD4^+CD25^+CD69^-$ and a potent suppressive effect in human tonsil GCs (25). Three groups independently discovered specialized $Foxp3^+$ Tregs constitutively expressing CXCR5, which can migrate into GCs and regulate the GC reaction (14–16). As CXCR5 is a Tfh-associated molecule and $Foxp3^+$ is usually expressed in Tregs, it is generally believed that Tfr cells have characteristics of both Tfh cells and Tregs. Other signaling molecules such as the transcription factors B-cell lymphoma 6 (Bcl-6), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), inducible T-cell costimulator (ICOS), and programmed cell death 1 (PD-1) are also expressed at different levels in Tfr cells (26). Indeed, Tfr cells have different phenotypes at different differentiation stages or in different locations. For example, mature GC-localized Tfr cells downregulate *Il-2Ra* (CD25) (27). The expression of interleukin (IL)-2 receptor *Il-2Ra* is also decreased in Peyer's patches (PP) Tfr cells, causing them to be unresponsive to IL-2 (28).

Circulating $CXCR5^+$ $Foxp3^+$ T cells (termed cTfr cells) have been described as the counterparts of tissue Tfr cells (tTfr cells) given that human tissues are unavailable (20, 21, 23). Compared to tTfr cells, little is known about the generation and functions of cTfr cells. cTfr cells are primed by dendritic cells (DCs) and have properties of naive memory cells. They express lower levels of ICOS than lymph node (LN) Tfr cells (29). In one study, cTfr cells even did not express ICOS, PD-1, or Bcl-6 (30). Similar to circulating Tfh, cTfr cells remain for a long time in blood and can be recruited into GCs. In addition, they have weaker suppressive capability than tTfr cells (29, 30). Hence, circulating memory-like Tfr cells are not canonical Tfr cells in terms of function and phenotype. Moreover, the generations of tTfr cells and cTfr cells are also different. The immunized μMT mice (lacking B cells) showed a reduced number of Tfr cells in draining LNs (dLNs) and an unchanged number of blood Tfr cells (29). This indicates that tTfr cells are more likely to develop in a B cell-dependent manner, while cTfr cells are not. Similarly, the frequency of blood Tfr cells is not decreased in B cell-deficient patients (30). It seems that cTfr cells (and cTfh cells) are likely generated when primary Tfr cells leave the LN without passing the B-cell zone, which might lead to incomplete cTfr cell suppression (30). Moreover, both CD28 and ICOS are required for the development of cTfr cells (18, 31). The differences and interplay between tTfr cells and cTfr cells warrant further study.

Tfr cells were initially thought to arise from natural (thymus-derived) Tregs (15, 16). Linterman et al. reported that 97% of Tfr cells express Helios (15). Helios is a transcription factor expressed by thymus-derived Treg cells (32). However, Tfr cells are not found in human thymus (16, 30) but are induced from natural Tregs in the periphery (16). One explanation is that the differentiation of Tfr cells requires multiple stimulations. The microenvironment of the thymus is required for Treg precursor cells to obtain initial molecules such as CD31 and Helios. The differentiation into mature Tfr cells is achieved by subsequent stimulation in peripheral lymphoid tissues (30, 33, 34). Interestingly, in mice, Tfr cells can be derived from naive $Foxp3^-$ precursors if adjuvant-promoting T-cell plasticity is used (35).

The differentiation of Tfr cells is a multistep process with various positive and negative regulators (**Table 1**). Early Tfr cell differentiation may be triggered by antigen presentation by DCs in secondary lymphoid organs (43). The antigen signals initiating Tfr and Tfh cell generation are unclear. Tfr cells differentiate after stimulation by foreign antigens (including ovalbumin and keyhole limpet hemocyanin), self-antigens (myelin oligodendrocyte glycoprotein), or viruses (43). Notably, Tfr cells are more responsive to self-antigens than to foreign antigens (39, 44, 45). This is supported by the fact that Tfr cells

prevent a self-reactive B-cell response but do not respond to the influenza-specific B-cell response (39). In addition, Tfr cell counts are higher in insulin (self-antigen)-immunized animals than in ovalbumin (foreign antigen)-immunized animals (45). T-cell receptor (TCR) repertoire analyses have suggested that Tfr and Tfh cells have different TCR repertoires (44). Indeed, the TCR repertoire of Tfr cells may be more similar to Tregs than to Tfh cells, consistent with the similar inhibitory functions of Tfr cells and Tregs (44). Adoption of the canonical phenotype by Tfr cells is likely dependent on interactions with cognate B cells in the GC. In B cell-deficient μMT mice, Tfr and Tfh cell development is abrogated after immunization (15). However, the intracellular signaling events involved in Tfr cell generation are incompletely understood. Notably, the chemokine receptor CXCR5 promotes the migration of Tfr cells into the GC (15, 16). The transcription factors NFAT2 and Bcl-6 upregulate the expression of CXCR5 (15, 16, 36), thereby indirectly promoting Tfr cells differentiation. In addition, Tregs lacking Bcl-6 expression cannot develop to Tfr cells. While loss of Blimp-1, a transcriptional repressor, downregulates Bcl-6 expression, it increases the number of Tfr cells. Given that, Bcl-6 may be required for Tfr cells generation, while Blimp-1 restrains the expansion of Tfr cells (through Bcl-6 inhibition) (15). Moreover, full Tfr differentiation relies on costimulatory molecules (CD28, ICOS), co-inhibitory molecules (PD-1), and cytokines (IL-2, IL-6, and IL-21) (15, 18, 29). Sage et al. (18) found that Tfr counts are very low in both LNs and blood of mice lacking CD28 or ICOS. CD28 may mediate the interaction with B cells and DCs in the process of Tfr generation because B7 (CD28 receptor) and ICOS ligand (ICOSL) are expressed in B cells and DCs (46). CD28 costimulation is required for Foxp3 expression during Treg differentiation (47). ICOS controls persistent Tfh cell migration into GCs in a manner independent of antigen presentation by DCs or cognate B cells (48). In addition, ICOS activates phosphoinositide-3-kinase (PI3K) signaling, which is crucial for the induction of Bcl-6, MAF, IL-4, and IL-21 (49), and therefore influences Tfh cell differentiation (50–52). These functions of CD28 and ICOS might also occur in Tfr cells.

Both Tfh cells and Tfr cells show high levels of PD-1 expression (15, 16). The impact of PD-1 in humoral immunity is complex. Although PD-1 suppresses the generation of long-lived plasma cells through reducing Tfh cell-associated cytokine production (53), blockade of PD-1 signaling pathway leads to enhanced humoral immunity (54, 55). Available data support PD-1 as a positive regulator of Tfr cell differentiation. In the lymph nodes of PD-1/PD-L1-deficient mice, the number of Tfr cells is decreased (18). Moreover, PD-1 inhibits differentiation and function of Tfh cells (18, 54). Interestingly, PD-L1 signaling might promote Foxp3$^-$ T precursors differentiating into Tfr cells under specific circumstances (35). CTLA-4, which is often expressed in Tfr cells and Tregs, also regulates the differentiation of Tfr and Tfh cells (56, 57). Deletion of CTLA-4 at the start of immunization results in increased numbers of Tfr and Tfh cells (57). In addition, CTLA-4-deleted Tfr cells do not suppress Tfh and B cells (57). The influence of CTLA-4 in Tfr cells seems to be related to IL-10 and/or B7 (57, 58). A recent study suggested that ICOS signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation (37). The role of FOXO1 in Tfr cells differentiation is largely unknown but might be related to the regulation of Bcl-6 expression. The expression of STAT3 in Tregs is also important for Tfr cell differentiation in the spleen and in Peyer's patches (PPs). Tfr cell differentiation were severely blocked in Stat3CD4KO mice after immunization (59). Moreover, enhanced IL-6/pSTAT3 signaling might promote Tfh generation and thereby lead to an imbalance in circulating Tfh cells and Tfr cells (22). Indeed, IL-6 is recently proposed to allow GC Tfh cell generation under sustained TCR stimulation without response to inhibitory IL-2 signaling by negatively regulating IL-2Rβ (CD122) expression (60). It is likely that tumor necrosis factor receptor-associated factor 3 (TRAF3) is a particularly crucial mediator of Tfr differentiation (61). In TRAF3$^{Treg-Ko}$ (TRAF3-deficient) mice, the generation of Tfr cells is impaired after SRBC immunization. In addition, TRAF3 regulates ICOS expression via the ERK-AP1 signaling pathway in Tregs (61) and possibly also in Tfr differentiation.

The mammalian target of rapamycin (mTOR) signaling pathway seems to regulate the generation and function of Tfr cells (62). It is reported that the conversion of Tregs into Tfr cells can be precluded when the PI3K-mTOR signaling pathway is inhibited by Roquin (through upregulation of Pten) (40). Furthermore, the development of Tfr cells is regulated by miRNAs such as the miR-17–92 clusters, miR-146a, miR-99, and miR-155 (42). Moreover, the differentiation of Tfr cells is under the control of several cytokines. For example, IL-2 inhibits Tfr development (39) even though it supports the generation and function of Tregs (63). IL-21 suppresses Tfr differentiation through activating STAT3 signaling in BXD2 autoimmune mice (24). This regulation of IL-2 and IL-21 on Tfr generation will be discussed below.

Most of the described studies regarding the biology of Tfr cells were based on mice. This raises the question of whether Tfr biology in mice is replicated in humans. Indeed, there are a few differences between mouse and human Tfr cells. For example, the maintenance of Tfh cells and Tfr cells in LNs does not require GC B cells in patients receiving rituximab (rituximab depletes GC B cells in the LNs) (64). By contrast, in mice, Tfr cells developed in a B cell-dependent manner (15). Moreover, given that Tfr cell differentiation is regulated by various cells, whether the above regulators directly or indirectly (via their effects on other immune cells involved in the process of Tfr differentiation) act in Tfr cells is unclear.

FUNCTIONS AND REGULATORY MECHANISMS OF TFR CELLS

Tfr cells in GCs play an important regulatory role. They suppress humoral immunity by inhibiting Tfh and B cells (14–16). Specifically, they suppress downstream effector responses in B cells, including CSR and somatic hypermutation rather than initial B-cell activation (65). Suppressed B cells show a lower metabolic level in *in vitro* cocultures, even in the absence of Tfr cells, suggesting that Tfr cells regulate B cells at the epigenetic level (65). Notably, this regulatory function requires participation

TABLE 1 | Factors involved in Tfr cell differentiation.

Molecules	Regulatory mechanism	Possible effect	References
Transcription factors			
Bcl-6	Promotes the expression of CXCR5	Enhance	(16)
Blimp1	Downregulates the expression of CXCR5	Inhibit	(15, 16)
NFAT2	Promotes the expression of CXCR5	Enhance	(36)
FOXO1	Inhibits ICOS expression	Inhibit	(37)
Costimulatory and coinhibitory signals			
CD28	Likely maintains FoxP3 expression	Enhance	(18)
ICOS	Mediates expression of Bcl-6	Enhance	(18)
PD-1	Binds to PD-1 ligand	Inhibit	(18)
Cytokines			
IL-21	Reduces the expression of CD25 by upregulating Bcl-6 in Tfr cells and thereby lowers responsiveness to IL-2	Inhibit	(24, 38)
IL-2	Upregulates Blimp-1	Inhibit	(39)
IL-6	Downregulates FOXP3 transcription factors; might activate STAT3 pathway	Unknown	(22)
miRNA			
miR-17∼92	Targets PTEN and enhances PI3K-Akt-mTOR signaling	Enhance	(40)
miR-146a	Might target ICOS and inhibit NF-κB signaling	Inhibit	(41)
miR-99	Represses mTOR signaling	Inhibit	(42)
miR-155	Suppresses SOCS1 (an inhibitor of IL-2R signaling in Treg cells)	Enhance	(42)

of Tfh cells. Tfr cells do not inhibit B cell proliferation in cocultures of Tfr cells and B cells (without Tfh cells) (26, 65). Whether the suppressive effect on B cells is a direct effect of Tfr cells or a consequence of Tfh cell dysfunction caused by Tfr cells is unclear. Furthermore, Tfr cells inhibit Tfh cell proliferation and the production of IL-4 and IL-21 (29, 65). IL-21 has a widely known function in the generation and maintenance of GCs and is essential in the development of RA (66). Sage et al. (43) also proposed that Tfr cells induce the death of B cells and Tfh cells by secreting granzyme B, as do Tregs.

The regulatory mechanism of Tfr cells depends on multiple factors and pathways (**Figure 1**), for example, the direct suppressor CTLA-4. Loss of CTLA-4 in Tfr cells results in defective suppression of antigen-specific antibody responses (56, 57). Tfr cells inhibit Tfh cell generation, IL-4 production by Tfh cells, and the expression of CD80 and CD86 on B cells in a CTLA-4-dependent manner, which is crucial for restraining the GC reaction (56). Moreover, transforming growth factor (TGF)-β production by Tfr cells serves as a crucial role in preventing Tfh cell accumulation, self-reactive B-cell activation, and autoantibody production (67). The role of IL-10 in Tfr cell suppression is not fully clarified. IL-10 is a pleiotropic cytokine that provides a survival signal to B cells and regulates antibody production (68). It is likely that Tfr cells inhibit IL-10 production by Tfh cells (29, 69), thereby suppressing aberrant GC responses. By contrast, the level of IL-10 mRNA is high in Tfr cells (15) and Tfr cell product IL-10 to support GC B cell proliferation and GC response in acute infection with lymphocytic choriomeningitis virus (LCMV) (70). IL-10 deficiency in B cells prevented immune tolerance, which results in decreased Tfr cells and increased IL-21 expression by Tfh cells (71). Moreover, Tfr cells express the IL-1 decoy receptor and the IL-1 receptor antagonist to suppress the activation of Tfh cells (72). Notably, the ability of Tregs to suppress the expansion and pathological activity of naive T cells is impaired in $TRAF3^{Treg-Ko}$ mice (61). The mechanism underlying this process and whether a similar phenomenon occurs in $TRAF3^{Tfrcells-Ko}$ mice need to be determined.

TFR CELLS IN RA

As in other autoimmune diseases, the development and progression of RA are unclear. The chronic inflammation in RA is associated with multiple immune cells (73). It has been confirmed that Tfr cells regulate the GC reaction in mice (14–16). However, the involvement of Tfr cells and their relationships with other cell subsets in human autoimmune disease, such as RA, are still poorly understood.

Evidence regarding the participation of Tfr cells in RA as well as other autoimmune diseases is conflicting. Niu et al. (22) revealed suppressed differentiation of Tfr cells in patients with RA, accompanied by high levels of PD-1 and IL-21, negative regulators of Tfr cell differentiation. The frequency of circulating Tfr cells is decreased and negatively related to autoantibody [immunoglobulin G (IgG), RF, anti-CCP] levels and disease activity in patients with RA (22, 23). A similar decrease in circulating Tfr cells in active RA was reported by Romão et al. (74). Liu et al. (23) showed that inactive patients with RA have significantly increased circular Tfr cells compared to healthy controls (HCs). Patients with stable-remission RA show increased activation of Tfr subsets along with enhanced inhibition of Tfr cells to Tfh cells (23), suggesting that an increased number of Tfr cells suppress autoimmunity in patients

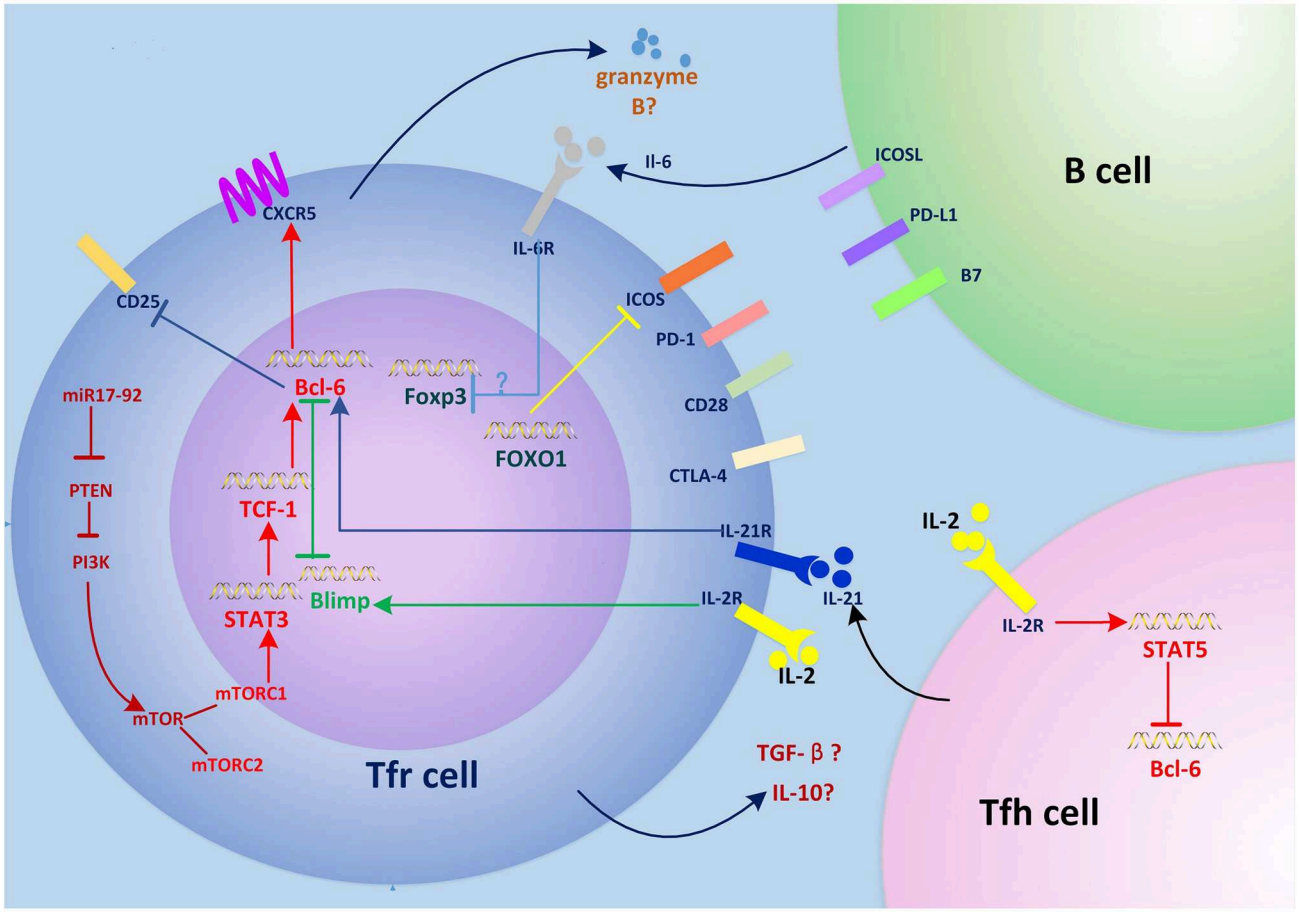

FIGURE 1 | Regulation of Tfr cell differentiation and function. The miR-17–92 clusters enhance PI3K-Akt-mTOR signaling by inhibiting PTEN, which promotes conversion of regulatory T (Treg) cells into T follicular regulatory (Tfr) cells. mTORC1 phosphorylates STAT3 and thereby induces expression of the transcription factor TCF1, which upregulates Bcl-6, inducing surface expression of CXCR5 and the migration of Tfr cells into germinal centers (GCs), where they regulate the GC response. Interleukin (IL)-6 signaling may downregulate FOXP3 and activate the STAT3 pathway at this stage. Interactions between Tfr cells and B cells such as ICOS-ICOSL and CD28-B7 are also required for Tfr cell generation. Tfr cells differentiation is inhibited by PD-1/PD-L1. IL-21 signaling also negatively regulates Tfr cell differentiation by promoting Bcl-6-mediated inhibition of CD25 (IL-2 receptor) and lowering responsiveness to IL-2. FOXO1 negatively regulates Tfr cell differentiation by inhibiting the expression of ICOS. Tfr cells have a suppressive effect on T follicular helper (Tfh) and B cells by secreting granzyme B, transforming growth factor (TGF)-β, and IL-10. The expression of CTLA-4 in Tfr cells inhibits this process.

with RA, stabilizing their condition. Importantly, restoration of the number of Tfr cells in mouse models inhibits aberrant immune responses. For example, transferring Tfr cells from BXD2-IL21$^{-/-}$ mice into young BXD2 mice suppresses the GC response and the production of autoantibodies (24). Lee et al. observed an increase in the number of Tfr cells in the spleen of collagen-induced arthritis (CIA) mice treated with intravenous immunoglobulin (IVIG), which was accompanied by a reduced serum level of RF (75). These studies suggest that the reduction of circulating Tfr cells is closely related to the development of RA and that restoration of the number of Tfr cells would ameliorate autoimmune responses. However, the increased number of Tfr cells in RA was also reported (76). Romão et al. (74) also found that inactive patients with RA and HCs have similar frequencies of circulating Tfr cells, which is not in line with Liu et al. In addition, the percentage of circulating Tfr cells in CD4$^+$ T cells was significantly reduced in an methotrexate (MTX) treated group compared to a non-treated group, suggesting that therapeutic schedule affects the frequency of Tfr cells. Moreover, similar increases in the number of circulating Tfr cells have been found in patients with other autoimmune diseases such as SLE, new-onset ankylosing spondylitis, and SS (30, 77–79).

Dysregulation of Tfh cells is strongly linked to the pathogenesis of autoimmune diseases (80–82). An increased number of circulating Tfh cells has been reported in patients with RA (23, 76, 83). Moreover, in patients with primary Sjögren's syndrome (SS), activated Tfh cells in peripheral blood correlate with disease activity (78). In one study, the frequencies of circulating CD86$^+$CD19$^+$ activated B cells and CD3$^+$CD4$^+$CXCR5$^+$ICOS$^+$PD-1$^+$ Tfh cells in patients with new-onset RA were higher than those in HCs and were positively correlated with disease activity (83). After treatment

with disease-modifying antirheumatic drugs (DMARDs) and a Chinese herb, the frequencies were reduced significantly (83). The increase in circulating Tfh cells also occurs in patients with active or inactive RA (23). However, Romão et al. (74) found that the frequency of Tfh cells in patients with inactive RA is less than that in HCs possibly due to the heterogeneity of patients.

The Tfr/Tfh ratios in RA are also altered. In BXD2 mice, a model of erosive arthritis mediated by excessive production of autoantibodies, the Tfh/Tfr and B/Tfr ratios in the spleen are significantly higher than those in wild-type (WT) mice (84). In addition, the Tfh/Tfr ratio is increased in the peripheral blood of patients with RA compared to that in HCs and is positively correlated with the DAS28 index (22). Indeed, Wang et al. (76) found that the circulating Tfr/Tfh ratio is inversely correlated with serum levels of CRP, erythrocyte sedimentation rate (ESR), RF, anti-CCP, IgG, and the DAS28 index. The Tfr/Tfh ratio is low in patients with RA, although the absolute numbers of Tfh cells and Tfr cells are higher, which suggests greater expansion of Tfh cells than Tfr cells in RA (76). Thus, at least part of the pathological mechanism of RA seems to involve aberrant expansion of Tfh cells and insufficient suppression by Tfr cells. Concordantly, a recent study showed that patients with RA in stable remission have a significantly higher blood Tfr/Tfh ratio than those with active RA and HCs (23).

The clinical significance of circulating Tfr cells, Tfh cells, and the Tfr/Tfh ratio needs to be confirmed because they may be markers for RA diagnosis and disease severity assessment. Heterogeneity of patients among studies may explain the controversy over the role of Tfr cells in RA. For example, patients in the study of Liu et al. (23) received no glucocorticoid and/or immunosuppressive drug within 1 month. In the study of Romão et al. (74), patients were treated with methotrexate, with or without other csDMARDs and/or glucocorticoids. It has been reported that methotrexate and DMARDs can significantly alter the number of circulating Tfr cells (76, 83). The controversy might be due to the use of different treatment regimens in patients (11). The definition of remission in RA is also inconsistent in these studies. The active disease group in Romão et al. (74) involves long-standing RA patients with DAS28 $>$ 3.2. But the active group is defined as any joint with active disease or any sign of systemic disease by Liu et al. (23). In addition, the development of RA is dynamic, and we speculate that the number of circulating Tfr cells differs according to disease duration. As stated by Deng et al. (11), Tfr cells and Tfh cells can be induced and expanded by self-antigen stimulation. However, the expansion of Tfh cells is greater than that of Tfr cells, probably leading to an excessive GC response in RA (11). We speculate that the increased number of Tfr cells in RA can be explained as a reaction to the excessive autoimmune response. On this assumption, the number of circulating Tfr cells is more likely to increase significantly in active RA patients than in patients with inactive RA. Hence, the Tfr/Tfh ratio has greater clinical significance than the absolute number of Tfr cells or Tfh cells. In addition, the different sample sizes of previous studies may also explain the diversity of their conclusions. Further studies need to take these factors into consideration and control sample heterogeneity.

Compared to the frequency of Tfr cells, much less is known regarding the functions of Tfr cells in RA. In Liu et al. (23), Tfr cells from peripheral blood of patients with RA in stable remission and HCs were cocultured with Tfh cells and B cells, respectively. They found that the suppressive effects of Tfr cells in RA with stable remission were enhanced (23). Moreover, circulating Tfr cells from patients with MS have a reduced suppressive effect compared to the equivalent cells from HCs (21). Similarly, Tfr cells in patients with RA might be functionally deficient. Notably, $FOXP3^+$ Tregs suppress ectopic lymphoid structure (ELS) generation in chronic inflammation (85). ELSs develop in the synovium of a minority of patients with RA (86), which contributes to RA by inducing local production of autoantibodies (87). Tfr cells have been found in the ELS in minor salivary glands of patients with SS, and the blood Tfr/Tfh ratio is associated with ELS formation in the minor salivary glands (78). Given that Tfr cells are also $FOXP3^+$, whether they suppress the generation of ELSs and the underlying mechanism warrant further study.

It is important to note that chronic inflammation in RA is associated with many other cell types, for example, B cells, monocytes, fibroblasts, and peripheral helper T cells (73). Rao et al. (88) found a population of PD-1^{hi} $CXCR5^-$ $CD4^+$ peripheral helper T cells (Tph) capable of promoting B-cell responses and antibody production through IL-21 and CXCL13 in the RA synovium. The frequency of Tph cells is significantly higher in seropositive RA synovial fluid than that in seropositive RA patients (88). The reduction of disease activity parallels the reduction in the frequency of Tph cells in RA after medication (88). Notably, Th17 cells, newly defined T effector cells, induce tissue inflammation and organ-specific autoimmunity by producing proinflammatory cytokines such as IL-17, IL-6, IL-21, and IL-22; all of these cytokines are involved in the pathology of RA (89, 90). The imbalance between Tregs and Th17 cells may be involved in autoimmunity (91). A recent study based on a large number of samples from inflamed joints revealed the potential of sublining synovial fibroblasts as a therapeutic target in RA. Four synovial fibroblast subpopulations were identified based on their major histocompatibility complex (MHC) II expression and cytokine production. Different synovial cell types seem to drive different inflammatory pathways in patients with RA and those with osteoarthritis (73).

The mechanism underlying the altered number of Tfr cells and Tfh cells in RA warrants further investigation. Given the issues with obtaining Tfh cells and Tfr cells from secondary lymphoid organs, most findings are based on circulating Tfr cells and Tfh cells. Whether similar changes in the number of Tfr cells and Tfh cells occur in the GCs of secondary lymphoid organs and synovial tissue of RA patients is not clear. Moreover, whether Tfh cells also regulate Tfr cells should be studied. Significantly, some seronegative patients with RA lack RF and anti-CCP in serum. This raises the question of what roles Tfr cells and Tfh cells play in seronegative patients with RA.

POTENTIAL TARGETS IN TFR CELLS

Modified Differentiation of TFR Cells via the mTOR Pathway

The mTOR pathway is a key regulator of the development of Tfr cells (40, 62). mTOR is a serine/threonine kinase that integrates various messages to dictate gene transcription and translation, as well as apoptosis, autophagy, and proliferation (92). There are two distinct complexes of mTOR, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (92). Tfr cells exhibit elevated mTORC1 signaling compared to Tregs after antigen stimulation (62). Current studies indicate that the mTOR pathway can regulate the development of Tfr cells. For instance, Roquin inhibits the PI3K-mTOR pathway at several levels, consequently inhibiting the conversion of Tregs into Tfr cells (40). The deletion of Rptor, an essential component of mTORC1, leads to impaired differentiation of Tfr cells in mice (62). This impairment is reflected in reduced levels of CXCR5, glucocorticoid-induced TNF receptor family-related protein (GITR), and CTLA4 in Tfr cells (62). Mechanistically, mTORC1 induces the expression of the transcription factor TCF1 by phosphorylating STAT3, which promotes expression of Bcl-6 in Tregs and upregulates CXCR5 in Tfr cells (62). These findings shed light on the importance of the mTORC1-pSTAT3-TCF-1 axis in the differentiation of Tfr cells. Moreover, mTOR pathway is also required for the function of Tfr cells. The expression of components of the mTORC1 pathway in B cells was reduced during suppression by Tfr cells (65). Differentiated Tfr cells showed attenuated function in suppressing the expansion of B cells when treated with rapamycin (a mTORC1 inhibitor) (62).

Evidences suggest that the mTOR pathway is a potential target against RA and other autoimmune diseases by restoring functional and numeric Tfr disorders. Baicalin, a natural compound isolated from Chinese herb, restores the balance of Tfh and Tfr cells by inhibiting the mTOR signaling pathway, leading to amelioration in lupus nephritis (93). The combination of an mTOR inhibitor and vitamin D3 prevented bone destruction in RA, as the PI3K/Akt/mTOR pathway is critical for osteoclast differentiation and survival (94). Rapamycin suppresses the erosion of fibroblast-like synoviocytes in the synovial tissue of patients with RA (95). As mentioned above, the mTOR pathway is involved in multiple biological processes in addition to regulating Tfr cell development; therefore, targeting mTOR to regulate Tfr cells may induce side effects. To achieve precise regulation of Tfr cells via the mTOR pathway, further studies need to clarify the underlying mechanisms. Identification of downstream targets in the mTOR signaling pathway or combinations of multiple targets might avoid unwanted effects and achieve a balanced Tfr/Tfh ratio in RA.

Regulation of the Differentiation and Function of Tfr Cells by Epigenetic Modification

MicroRNAs (miRNA) are regulatory small non-coding RNAs, which play an important role in gene expression, particularly at the posttranscriptional level. miRNAs regulate the immune response by suppressing the expression of key immune-associated genes (96, 97). In genome-wide expression analyses, miR-146a is highly expressed in human Tfh cells, and miR-146a deficiency leads to the accumulation of Tfh cells and GC B cells. Mechanistically, miR-146a suppresses the responses of Tfh cells and GC by repressing ICOS signaling (41). Moreover, the miR-17–92 cluster acts as a crucial regulator of Tfh cells by promoting the differentiation of Tfh cells. In mice with miR-17–92-deficient T cells, the number of splenic Tfh cells was significant decreased (98). Overexpression of the miR-17–92 cluster in T cells in lymphocytes leads to autoimmunity in mice (99). However, the role of miR-17–92 in the development of Tfr cells is unclear. Although overexpression of miR-17–92 in $CD4^+$ T cells results in an increased frequency of Tfr cells (98), it has been proposed that miR-17–92 suppresses Tfr cell generation in mice with chronic graft-versus-host disease (100). A possible mechanism by which miR-17–92 regulates Tfr cells is targeting Pten (a PI3K inhibitor) in Tregs, thereby enhancing PI3K-Akt-mTOR signaling (40). Other miRNAs implicated in Tfr cell differentiation are summarized in **Table 1**. Given the impact of miRNAs in Tfr and Tfh cell development, it is likely that miRNAs could be a regulator in Tfr/Tfh balancing during RA development. Notably, miRNA-based treatments are effective in several autoimmune diseases. For example, in a mouse model of lupus-like chronic graft-versus-host disease, inhibitors of miR-17 alleviate clinical manifestations (100). Thus, it is likely that miRNA clusters that promote Tfr cell generation and inhibitors of miRNA clusters that negatively regulate Tfh cells could restore the Tfr/Tfh balance in RA patients with a decreased Tfr/Tfh ratio.

Epigenetic modification by histone deacetylase (HDAC) is closely related to chronic inflammation and autoimmunity (101, 102). HDAC regulates cell differentiation or death at the transcriptional level (103) and possesses anticancer activity (104). Moreover, HDAC9 deficiency leads to decreased autoantibody production and Bcl-6 expression in MRL/lpr mice (101). In models of colitis, strategies to reduce HDAC9 expression enhance the function of Tregs and prevent colitis (105). Thus, we speculate that HDAC inhibitor might also regulate the function and/or generation of Tfr cells at the transcriptional level because the phenotype and function of Tfr cells are similar to those of Tregs. HDAC inhibitors (HDACi) suppress the expression of IL-4, interferon (IFN)-γ, TNF-R, and IL-7R and are used in organ or bone marrow transplantation to induce immune tolerance (106). Further studies are needed to address whether HDACi regulates the expression of IL-21, Bcl-6, and other molecules involved in Tfr cell development.

Tfr Cells and the Gut Microbiota

The microbiota have been investigated intensively in recent years given its profound impact on immunity. The microbial communities, their metabolites, and components may act as pathogen-associated molecular patterns (PAMPs), which are detected by pattern recognition receptor and activate antigen-presenting cells to initiate an immune response (107, 108). However, little is known about the mechanism by which the gut microbiota modulate autoimmune diseases.

The gut microbiota have both anti-inflammatory and proinflammatory functions. Segmented filamentous bacteria (SFB) promote the generation of Th17 cells in the gut and induce autoimmune arthritis in K/BxN mice (109). They also induce PP Tfh cell differentiation and migration into systemic sites, leading to autoimmune arthritis (110). By contrast, capsular polysaccharide A of *Bacteroides fragilis* induces the differentiation of $CD4^+$ T cells into Tregs, thereby suppressing inflammation (111, 112). Moreover, butyrate, a common intestinal metabolite, promotes Treg differentiation (113). Tfr cells are derived from Tregs, and the gut microbiota might affect Tfr cell differentiation by regulating Tregs. Notably, short chain fatty acid (SCFA) produced by the microbiota modulates the production of antimicrobial peptides, which regulate intestinal homeostasis by activating mTOR and STAT3 in intestinal epithelial cells (IECs) (114). As mentioned above, the mTORC pathway is involved in the differentiation of Tfr cells. Therefore, microbial metabolites might regulate the Tfr/Tfh balance to induce immune tolerance through the mTORC pathway. Moreover, Tfr cells also have an impact on gut microbiota. $Foxp3^+$ T cells facilitate diversification of the gut microbiota, but this is suppressed by selective inactivation of Bcl-6 (115). Because $Foxp3^+$ T cells differentiate into Tfr cells in a Bcl-6-dependent manner, Tfr cells likely contribute to maintenance of the diversity of the gut microbiota.

Importantly, regulation of the gut microbiota can alleviate RA. In a mouse model, *Lactobacillus* reduces pannus formation, synovial infiltration, and bone destruction (116). In a clinical trial, patients with RA who consumed *Lactobacillus casei* for 8 weeks showed a significant decrease in disease activity score, along with reduced serum levels of proinflammatory cytokines such as tumor necrosis factor-α, IL-6, and IL-12 (117). As mentioned above, these cytokines are involved in the development of Tfr cells.

Altogether, cross talk between Tfr cells and gut microbes such as SFB, *B. fragilis*, and *Lactobacillus* may be involved in the development of RA, which warrants further exploration.

The IL-2–IL-21 Axis in the Tfh and Tfr Cell Balance

IL-2, a pleiotropic cytokine mainly produced by $CD4^+$ T cells after antigen stimulation, has a function in maintaining the generation and function of Tregs (118, 119). Tregs suppress GC B-cell responses and autoantibody production (120). And Foxp3 is required for the development of Tregs (121, 122). IL-2 upregulates Foxp3 expression in Tregs via Jak/STAT5 signaling and thereby promotes Treg-mediated immune suppression *in vivo* (123, 124). Tregs are sensitive to IL-2, as indicated by their higher expression of *Il-2Ra* than other Teff cells (125). It is generally believed that low-dose IL-2 selectively activates and promotes the generation of Tregs (126). Interestingly, the number of Tregs in the thymus is increased in $Il\text{-}2Ra^{-/-}$Tg mice, but their suppressive effect is decreased (63). In contrast, peripheral Tregs have an unchanged suppressive effect *in vitro* and activated Th1-like phenotype (63). Importantly, low-dose IL-2 inhibits the generation of human Th17 in SLE and pSS (127–129). Related to promoting Treg generation and/or suppressing Th17 differentiation, low-dose IL-2 has been known as a strategy to ameliorate various immune-mediated disorders (127, 130).

IL-2 inhibits the differentiation of Tfh cells *in vivo* (131). The number of Tfh cells is positively correlated with the intensity of the humoral response (132). Mechanically, IL-2 represses Bcl-6 expression via the STAT5 pathway, which precludes the differentiation of Tfh cells and so controls the GC reaction (133, 134). Bcl-6 is critical for Tfr cell differentiation (16). Given the repressed expression of Bcl-6 by IL-2 in Tfh cells, low-dose IL-2 might inhibit Tfr cell generation. Botta et al. (39) showed that high concentrations of IL-2 at the peak of the infection preclude Treg development into Tfr cells by promoting Blimp-1, an essential suppressor in Bcl-6 expression. In addition, Bcl-6 and CXCR5 are upregulated, which is required for development into Tfr cells, in $Foxp3^+$ cells cultured in the presence of a low level of IL-2. Interestingly, Tfr cells with different origin showed different responsiveness to IL-2. PP Tfr cells have a robust downregulation of *Il-2Ra* and remained largely unresponsive to IL-2, while the expression of *Il-2Ra* on Tfr cells of pLN origin is high, suggesting that IL-2 signaling pathway is operative in these cells (28).

Although IL-2 negatively regulated Tfr cell differentiation, blocking TGF-β and IL-2 signaling disrupts Treg phenotype and function as well as their differentiation into Tfr cells. This leads to an increased number of Tfh cells and enhanced GC response (63). Thus, IL-2 plays an important role in the development of Tregs and Tfr cells. Disruption of the role of IL-2 in Tfr, Tfh, and Treg cells development might be involved in RA. The pathway by which IL-2 regulates Tfr cell differentiation and how IL-2 influences the function of Tfr cells in RA need to be defined.

IL-21, mainly produced by Tfh cells, modulates the GC reaction by counteracting the suppression of Treg and promoting the production of antibodies (135, 136). Blockade of IL-21 reduces disease severity in mice with RA, indicating that IL-21 is closely related to RA progression (137). In addition, IL-21 promotes Tfh cells but inhibits Tfr cell development in BXD2 mice (24). Sage et al. (65) found that high concentrations of IL-21 overcome Tfr cell-mediated suppression of B cells. Consistent with this, IL-21 reinforces the humoral immune response by inhibiting the proliferation of Tfr cells (38). Mechanically, IL-21 signaling has a negative effect on the expression of CD25 by upregulating Bcl-6, which reduces responsiveness to IL-2 and decreases the proliferation of $CD25^+$ Tfr cells (38).

These findings suggest that IL-21 and IL-2 regulate Tfr development and function, and their balance is key for maintaining a normal Tfr/Tfh ratio. Indeed, impaired IL-2 production, a high IL-21 concentration, and an aberrant Tfr/Tfh ratio are typically found in patients with RA (22, 76, 138). In the CIA model, blockade of the IL-21 pathway ameliorates disease (137). In SLE patients, low-dose IL-2 treatment modulated $CD4^+$ T-cell subsets and reduced disease activity (127). The mechanisms by which IL-21 and IL-2 regulate inflammation via Tfr cells and Tfh cells remain to be determined; such information could help prevent RA.

CONCLUSIONS AND FUTURE PERSPECTIVES

Tfr cells are critical mediators of GC response with great therapeutic potential in RA, although their role in autoimmune diseases is controversial. Regulation of Tfr cells requires various regulators such as mTOR signaling, HDACi, microRNA, cytokines, and the gut microbiota. These regulators are closely linked and largely unclear. For instance, miR-17–92 targets PTEN, thus enhancing PI3K-Akt-mTOR signaling (40). SCFAs are important HDACi (113). Indeed, RA is a complex autoimmune disease with multiple regulatory mechanisms. The therapy only by Tfr cell regulation in RA might have limitations. Combinations of multiple strategies could pave the way for the development of immunotherapies for RA. It is important to systematically assess the correlation between the number of circulating Tfr cells and markers of the diagnosis, severity, treatment efficacy, and prognosis of RA. Moreover, the relationship among circulating Tfr cells, tissue Tfr cells, and PP Tfr cells should be clarified. We believe that Tfr cells represent an attractive therapeutic target in RA and warrant further mechanistic studies and clinical trials.

AUTHOR CONTRIBUTIONS

TD drafted the manuscript, prepared illustrations, and discussed the content with the other authors. CW conceived the topic and revised the manuscript. HN intellectually revised the manuscript. XZ, CG, and XL also critically revised the manuscript for intellectual content. All of the authors approved the manuscript for publication.

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REVIEW
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Getting to the Heart of the Matter: The Role of Regulatory T-Cells (Tregs) in Cardiovascular Disease (CVD) and Atherosclerosis

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Cardiovascular diseases (CVD) are the leading cause of mortality worldwide. Atherosclerosis is directly associated with CVD and is characterized by slow progressing inflammation which results in the deposition and accumulation of lipids beneath the endothelial layer in conductance and resistance arteries. Both chronic inflammation and disease progression have been associated with several risk factors, including but not limited to smoking, obesity, diabetes, genetic predisposition, hyperlipidemia, and hypertension. Currently, despite increasing incidence and significant expense on the healthcare system in both western and developing countries, there is no curative therapy for atherosclerosis. Instead patients rely on surgical intervention to avoid or revert vessel occlusion, and pharmacological management of the aforementioned risk factors. However, neither of these approaches completely resolve the underlying inflammatory environment which perpetuates the disease, nor do they result in plaque regression. As such, immunomodulation could provide a novel therapeutic option for atherosclerosis; shifting the balance from proatherogenic to athero-protective. Indeed, regulatory T-cells (Tregs), which constitute 5-10% of all $CD4^+$ T lymphocytes in the peripheral blood, have been shown to be athero-protective and could function as new targets in both CVD and atherosclerosis. This review aims to give a comprehensive overview about the roles of Tregs in CVD, focusing on atherosclerosis.

Keywords: regulatory T cells (Tregs), cardiovascular disease (CVD), hyperlipidemia, hypertension, atherosclerosis

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INTRODUCTION

According to the World Health Organization [https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)], cardiovascular diseases (CVD) are a group of disorders affecting the heart and blood vessels; which include coronary heart disease (ischemic heart disease), angina, myocardial infarction, congenital heart diseases (e.g., tetralogy of Fallot, ductus arteriosus, transposition of great vessels, tricuspid atresia), hypertension, stroke (e.g., ischemic or hemorrhagic), heart valve diseases (e.g., regurgitation or stenosis), cardiomyopathy (e.g., heart failure with dilated or hypertrophic cardiomyopathy or with preserved ejection fraction), and vascular dementia.

Atherosclerosis is a common cause of CVD and is characterized by slow progressing inflammation in conductance and resistance arteries, in which there is an accumulation of cholesterol-containing low-density lipoprotein (LDL) particles beneath the endothelial layer (1). These lipid accumulations often take several decades to become symptomatic. Lesions associated with the disease can be found in the aorta as early as the first decade of life. Aging, genetic, and environmental factors lead to the spread of these lesions, they can be found in sites such as coronary arteries in the second decade of life, and cerebral vessels in the third and fourth decades of life (1).

Hypertension and hyperlipidemia are key risk factors for arteriosclerosis. As a result, statins (HMG CoA reductase inhibitors) have been widely used in CVD patients (2). Meta-analysis studies have suggested that for each 1 mmol/L reduction in LDL levels corresponds to a 22% decreased in the risk of stroke and coronary heart disease (3). However, data from surveys, registries, and insurance claims indicate that adverse effects of statins are common, which discourage patients from continuing therapy at recommended doses. Additionally, the underlying chronic inflammation of the blood vessel(s) is not completely resolved by statins.

Several studies have shown that the immune system is activated in atherosclerosis. Such observations indicate the possibility that selective suppression of proatherogenic or activation of athero-protective immune mechanisms may represent novel approaches for disease treatment. In recent years regulatory T-cells (Tregs) have emerged as crucial players in modulating both the innate and adaptive immune responses (4). Impaired Treg function and decreased frequency has been associated with the progression of atherosclerosis (5–7). Furthermore, adoptive transfer of such cells in animal models for atherosclerosis has been shown to be protective (7). Therefore, Tregs could be important targets in atherosclerosis and understanding their functions in this context is fundamental to driving future therapies.

PATHOGENESIS OF ATHEROSCLEROSIS

The pathogenesis of atherosclerosis is illustrated in **Figure 1.** The disease is initiated by the passive diffusion of LDL into the arterial intima, this occurs preferentially in regions of higher blood turbulence or parts where the sites of endothelial damage (1, 8). Following diffusion into the sub-endothelial space, LDL is able to bind proteoglycans via apolipoprotein B-100 (ApoB100), and subsequently becomes permanently retained (9). The sequestered LDL undergoes oxidative modification forming oxLDL which causes aggregation and increased proteoglycan binding (8). Oxidation of LDL is mediated by reactive oxygen species (ROS) produced by smooth muscle cells (SMCs), endothelial cells (ECs), neutrophils, and macrophages (9, 10). These events are potentiated by production and release of monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (m-CSF), which, respectively, attracts circulating monocytes to the plaque and activates them to release more ROS, nitric oxide (NO), and pro-inflammatory cytokines, such as TNF-α and IL-1β (1, 11). In a positive feedback loop, ROS induces expression of TLRs in ECs, which perpetuates the inflammatory response via the expression of adhesion molecules, which cause circulating monocytes and other leukocytes to enter the tissue via trans-endothelial migration. Monocytes become differentiated into macrophages (8) which, once present within intima layer of the arteries, engulf oxLDL (8) through the scavenger receptors SR-A and CD36 (1). This uptake leads to the formation of foam cells, which have compromised migratory capacity. Consequently, these cells accumulate in the intima and die, resulting in the formation of a plaque with a necrotic core (1).

Growth factors and cytokines released by ECs and macrophages induce multiple effects including phenotypic changes within vascular SMCs, from the quiescent "contractile" phenotype state to the active "synthetic" state, that can migrate to and proliferate within the intima. The migratory and proliferative capacities of VSMC's increase the size of the atherosclerotic plaque. Some of the emigrated VSMCs become less differentiated, senescent, or undergo apoptosis, which contributes to plaque instability and rupture (12). This leads the formation of a traveling thrombus which can occlude smaller arteries, resulting in myocardial infarction (MI) or ischemic stroke (13).

ATHEROSCLEROSIS AND T-CELLS

Both the adaptive and innate arms of the immune system are involved in the development of atherosclerosis. As demonstrated in **Figure 1**, innate responses occur first in a non-antigen-specific manner (14). However, modified self-molecules such as oxidized LDL (oxLDL), β2 glycoprotein 1, Lipoprotein A (LP(a)), heat-shock proteins (HSP), and glycosylated proteins from the blood vessels extracellular matrix (collagen and fibrinogen) have been described as antigens, thus activate T-cell responses during atherosclerosis (9). Furthermore, foreign antigens including bacteria such as *Porphyromonas gingivalis* (15) and *Chamydia pneumoniae* (16), and viruses such as enterovirus (17) and cytomegalovirus have also been associated with atherosclerosis; potentially as causative or bystanders participants, adding yet another layer of intricacy to fully understanding the pathophysiology of atherosclerosis.

Antigen presentation by dendritic cells (DCs) during atherosclerosis is a complex matter. Early research, which aimed to elucidate the role of DCs in atherosclerosis relied on CD11c as the identifying cell surface marker. Functionally, high-phagocytic activity was demonstrated for $CD11c^{-}CD11b^{+}MHCII^{+}$ macrophages that efficiently engulfed lipids, whereas $CD11c^{+}MHCII^{+}$ DCs present in the aorta were shown to display strong immune stimulatory capacities; being pivotal for T-cell activation and inflammation. However, in recent years it has become apparent that CD11c can also be expressed by $MHCII^{+}$ monocytes and macrophages, and an ontogenetic view of cell lineages has defined DCs as a hematopoietic lineage distinct from other leukocytes (18). Thus historic studies addressing the role of $CD11c^{+}$ ($MHCII^{+}$) DCs may have also unintentionally included other cell types, such as monocytes/macrophages, therefore the term APC may have been more appropriate (19).

APCs travel to draining lymph nodes and present antigens for recognition by T-cells (14). Such antigen recognition results

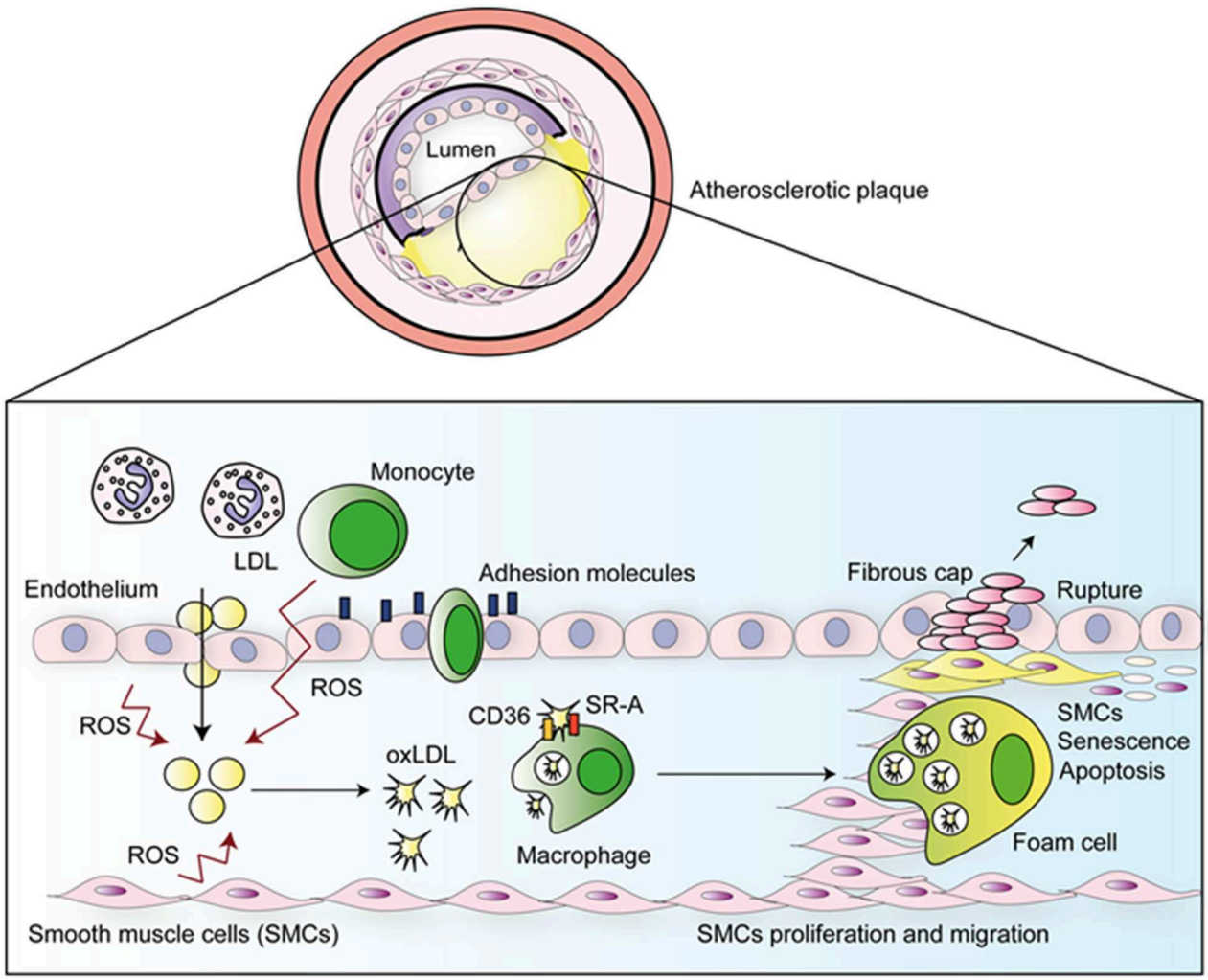

FIGURE 1 | Atherosclerotic disease progression. ROS produced by ECs, SMCs, neutrophils, and macrophages oxidized LDL in the sub-endothelial space. Emigrated monocytes become macrophages which uptake oxLDL, subsequently forming foam cells, that are unable to migrate to arterial lumen, accumulate in the sub-endothelial space and die forming a large plaque with a necrotic core. Rupture of this plaque leads to thrombus formation.

in clonal expansion of both $CD8^+$ and $CD4^+$ T-cells. $CD4^+$ T-cells can secrete cytokines such as IL-17 (20) and IFN-γ (21), which facilitate the inflammatory process. Furthermore, IL-4 and IL-13 production by $CD4^+$ T-cell activation leads to B-cell activation, clonal expansion, and subsequent immunoglobulin production (14). Antibodies appear to play a prominent role in atherosclerosis, arising due to increased number of immunogenic neo-epitopes which are typically present in the disease (14).

The atherosclerotic plaque consists of a heterogeneous population of cells, debris and extracellular matrix components (1). $CD4^+$ T-cells can be divided in different subsets according to their capacity to support the type of immune response and cytokine production. Th1 cells are the most abundant T-cells and in the context of atherosclerosis, promote disease progression (1). These cells secrete IFN-γ which promotes lesion development and destabilization leading to the plaque rupture (1). Additionally, IFN-γ activates monocytes causing continuation of the response. Th2 and Th17 cells have also been found in atherosclerotic lesions but at lower frequencies. Th2 cells release IL-4, IL-5, IL-13, and support B-cell activation and antibody production (21). IL-4 induces the expression of the transcription factors GATA-3 via STAT-6 activation, and stimulates Th2 cell differentiation leading to upregulation of IL-5 which inhibits Th1 differentiation and therefore IFN-γ production (8, 21). As a result of this regulatory role Th2 cells were initially assumed to be beneficial in the setting of atherosclerosis. However, recent evidence indicates that these cells may be both helpful and disadvantageous depending on disease stage and/or lesion site. Th17 cells produce IL-17 which is a pro-inflammatory mediator. Th17 development is promoted by TGFβ in the presence of IL-6 and IL-23 (21). Similar to Th2, Th17 cells have been reported to have both positive and negative roles in atherosclerosis (21). Th17 cells produce factors such as IL-6, IFNγ, and granulocyte-macrophage colony-stimulating

factor (GM-CSF), which are proatherogenic. However, opposing this proatherogenic role, Th17 cells can also convert into other cell types such as Tregs; by gaining expression of forkhead box P3 (FOXP3) and can subsequently exert suppressive effects on effector Th1 and Th2 cells.

REGULATORY T-CELLS (TREGS)

Tregs have been shown to be present within atherosclerotic plaques (22). Human Tregs were initially characterized as $CD4^+CD25^+$ T-cells in 2001 by several groups (23–26) based on the 1995 finding that murine Tregs constitutively express CD25 (27). These adaptive immune cells comprise 5–10% of all peripheral $CD4^+$ T-cells (28), and play an indispensable role in the adaptive immune system being responsible for both immune homeostasis and maintaining self-tolerance (4).

TREG DEVELOPMENT

Tregs can be sub-divided into two main classes depending on their developmental origin: thymic Tregs (tTreg) and peripherally induced Tregs (pTreg). tTregs develop in the thymus, an environment where tTregs with a high affinity for self-antigens are positively selected for maturation (29). Once active, these cells can migrate out of the thymus and into the peripheral tissues and lymph nodes (28). Conversely, pTregs develop via antigenic stimulation from conventional $CD4^+$ T-cells in the periphery.

SUBSETS OF pTregs

There are subsets of Tregs generated in the periphery which have specific phenotypes and a mechanism of action which is cytokine-dependent: Tr1 and Th3. Unlike tTregs, which arise as a separate sub-lineage from T-cell precursors in the thymus, Tr1 and Th3 derived from conventional peripheral Th0 cells and interact with and are susceptible to modulation by dendritic cells (30).

Tr1 cells are a population pTregs which are induced by sustained TCR engagement via chronic antigenic stimulation in the presence of high levels of IL-10. These cells act to induce and maintain peripheral tolerance. They do not exhibit constitutive FOXP3 expression, instead its expression is induced upon activation. Tr1 cells produce predominantly IL-10, whereas Th3 (also known as Tr2) predominantly produces TGF-β (30). Th3 cells differentiate in an antigen-non-specific manner and are identifiable via their expression of latency-associated peptide (LAP), IL-4 production and low CD25 expression, moderate levels of GITR and CTLA-4. Tr1 cells can produce IFN-γ and do not express detectable levels of GITR and low levels of CTLA-4 and CD25 (30).

Human Tregs consist of a heterogeneous population; characterized by the expression pattern of a vast range of cell surface molecules (27). Despite this variation, suppressive Tregs share the expression of certain "common" surface molecules such as CD4, CD25, and FOXP3 (31). FOXP3 is the transcription factor which is considered to be the lineage defining molecule, it's essential for both cell maturation and function (4).

SUPPRESSIVE MECHANISMS

The key role of Tregs is to suppress both the adaptive and innate immune system. Tregs achieve this via the utilization of both direct and indirect pathways. In a direct manner, Tregs themselves elicit an immune response upon a target cell by for example, the secretion of suppressive cytokines such as IL-10, TGFβ, and IL-35 (23). In an indirect way by expressing higher levels of CD25, Tregs compete with T-effector cells (Teffs) for IL-2, which limits their proliferation. Tregs also express CD39/CD73 on their surface which produces adenosine from ATP, and activates adenosine receptors A2 on Teffs, which has inhibitory properties (23). **Figure 2** gives examples of Treg suppressive mechanisms which are relevant in context of atherosclerosis.

EVIDENCE IMPLICATING TREGS IN ATHEROSCLEROSIS

There has been a growing interest about the role about Tregs in atherosclerosis, with many studies aiming to better understand their significance. In 2006 using the $ApoE^{-/-}$ mouse model, Ait-Oufella et al. demonstrated that $CD4^+CD25^+$ Treg deficiency is associated with a significant increase in atherosclerotic lesion size (5). This indicated for the first time that endogenous $CD4^+CD25^+$ Tregs play a protective role in atherogenesis (5). Further evidence came the following year, in 2007 Mor et al. (6) demonstrated that the adoptive transfer of wild type (WT) Tregs into $ApoE^{-/-}$ mice resulted in a significant reduction in aortic sinus plaques as compared to the control mice (6). Furthermore, Tregs from $ApoE^{-/-}$ mice we shown to have decreased *in vitro* suppressive ability (6). Then, studies in mice strongly suggest that defective Tregs may enable disease progression.

In 2013 Kligenberg et al. (7) demonstrated that deletion of $FOXP3^+$ Tregs results in a 2.1-fold increase in plaque size in mice. FOXP3 was selectively depleted in the presence of diphtheria toxin using the DREG system in $LDLR^{-/-}$ mice. This selective depletion resulted in increase in plasma cholesterol and VLDL levels and enhanced plasma enzyme activity of lipoprotein lipase, hepatic lipase, and phospholipid transfer protein (7). Importantly, in addition to induced changes within the cellular composition of the atheroma, Treg depletion also resulted in differences in genes controlling lipid metabolism in the liver and decreased the liver levels of sortilin, which may contribute to impairing intracellular cholesterol transport and increase the plasma levels of VLDL. Such results suggest that under normal conditions Tregs positively modulate VLDL cholesterol levels (7).

In summary, these studies demonstrated a link between Tregs depletion, decreased suppressive capacity and the development of atherosclerosis in mice. However, these studies failed to address key questions surrounding the Tregs roles in humans: what are the specific functions of Tregs in atherosclerosis? Why are Tregs defective in both number and

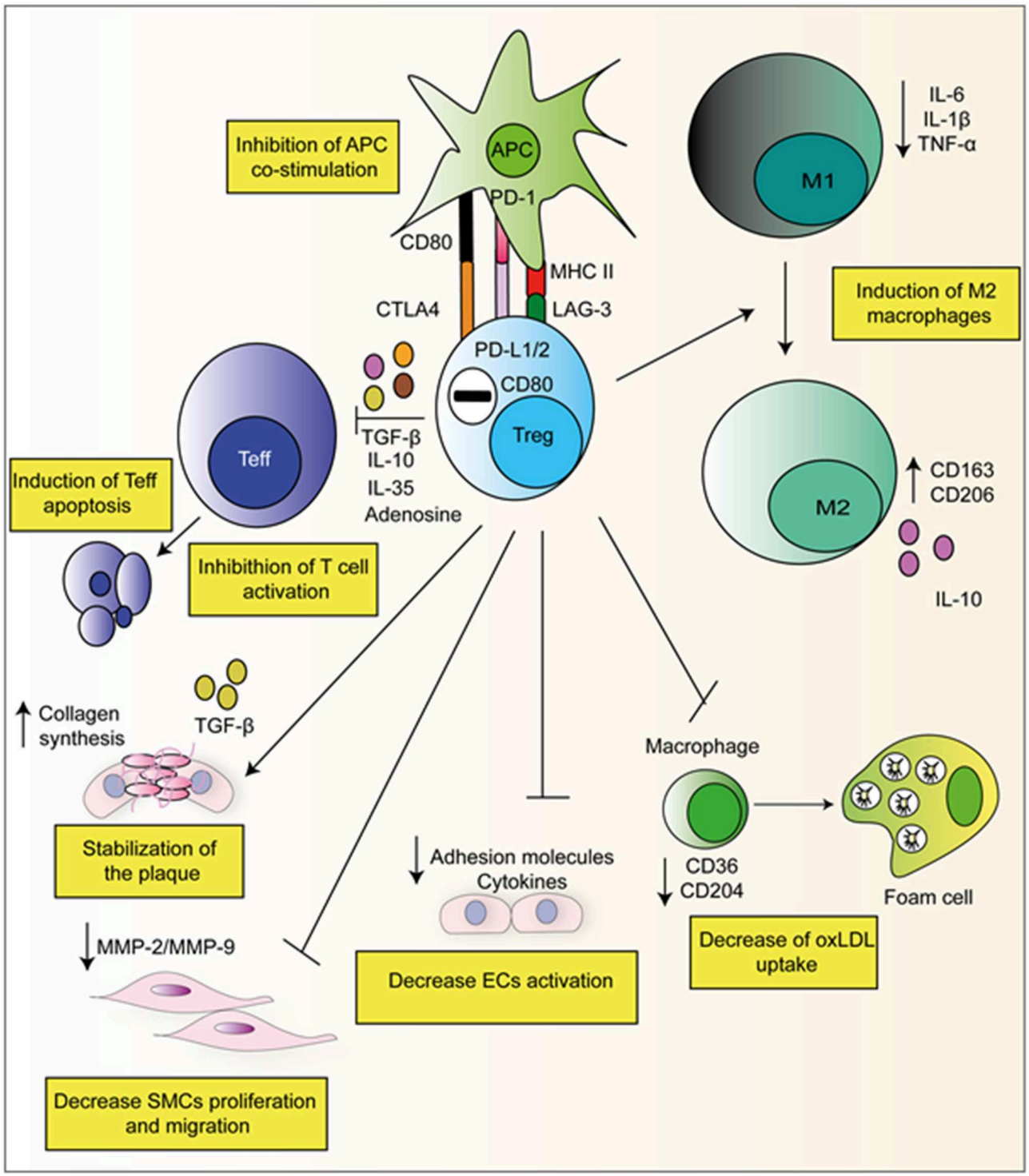

FIGURE 2 | Suppressive mechanisms of Tregs in atherosclerosis. Tregs can directly produce suppressive cytokines and degradative enzymes such as perforin and granzyme that leads to apoptosis. Furthermore, Tregs have been observed to have direct effects on APC's via interaction with via CTLA-4, PD-L1/2, and LAG-3. They can also skew monocyte class switching, encouraging anti-inflammatory M2 macrophages formation which produced collagen and stabilizes the plaque. They can also decrease foam cell formation via the down-regulation of CD36 and CD204.

suppressive function in atherosclerosis? And, most importantly, are the findings obtained in animal models applicable to humans?

THE FUNCTION OF TREGS IN ATHEROSCLEROSIS

Subsequent studies began to address the role of Tregs within atherosclerotic pathophysiology. Tregs suppress proatherogenic Th1 effector T-cells **(Figure 2)**. Studies in mice demonstrated the ability of Tregs to reduce the proliferation of Th1 and decrease IFNγ production via IL-10 synthesis (32). This finding has been mirrored in human CVD; compared to healthy control, patients suffering from CVD (stable angina, unstable angina, and acute MI) have a reduced $CD4^+CD25^+FOXP3^+$ population whilst simultaneously exhibiting an increased Th1 population in circulation (33). Furthermore, another study showed the presence of increased numbers of Th17 cells and a lower proportion of Tregs present in patients with unstable carotid artery legions (34), suggesting Tregs could suppress proatherogenic Th17 effector T-cells. The existence of these correlations suggests the existence of a shifted balance between anti-inflammatory Tregs and pro-inflammatory Th17/Th1 in patients with atherosclerosis, favoring the latter subsets.

Tregs have also been demonstrated to act directly on monocytes inhibiting their cytokine secretion, differentiation, and antigen-presenting function. Following co-culture with Tregs, monocytes exhibit classical features of M2 macrophages such as increased CD206 (mannose scavenger receptor) and CD163 (hemoglobin scavenger receptor) expression. Simultaneously, macrophages co-incubated *in vitro* with Tregs exhibited a reduced capacity to respond to pro-inflammatory LPS as demonstrated by both decreased production of IL-6 and TNF-α and decreased NF-kB activation (**Figure 2**) (35). Furthermore, our group has recently reported that *ex vivo* expanded Tregs are very efficient at skewing monocytes toward a M2 tolerogenic phenotype. Of note, monocytes co-cultured with expanded Tregs showed a reduced capacity to increase detrimental IL-17 producing T-cells as compared to freshly isolated Tregs (36). This mechanism resulted from the decreased CD86 expression by Treg-conditioned monocytes. In addition to suppressing effector T-cells and favoring M2 macrophage development Tregs have been previously shown to decrease foam cell formation via downregulating both SRA and CD36 (37).

Tregs also exert effects on APCs by inhibiting antigen presentation. Tregs can inhibit APC function by the expression of cell surface molecules such as CTLA-4 and PD-L1/2. CTLA-4 expressed by Tregs, binds to and trans-internalize CD80/CD86 from APCs, diminishing the ability of APCs to co-stimulate T-cells (38). Increased mRNA levels of CTLA-4 have been associated with increased Tregs and decreased atherosclerosis (39, 40). Signaling via the co-inhibitory PD-1 (on Tregs) and PD-L1/2 (on APCs) also inhibits their activation. Mice globally deficient in either PD-1 or PD-L1/2 show aggravated atherosclerosis mediated by increased effector T-cell responses (41).

In addition to their effects on other leukocytes, Meng et al. (42) demonstrated $ApoE^{-/-}$ mice adoptively transferred with Tregs had increased plaque stability, reducing the risk of plaque rupture by inducing collagen synthesis by M2 macrophages (42). Tregs have also been shown to suppress EC activation and cholesterol metabolism. pTregs suppress both TNFα and IL-1β mediated E and P-selectin expression by ECs (43).

Tr1 AND Th3 IN ATHEROSCLEROSIS

The role of Tr1 or Th3 cells in atherosclerosis and therefore their therapeutic potential is currently unclear. To date two studies using distinct mice models have evaluated the role of Tr1 in the pathogenesis of atherosclerosis.

Although no improvement in disease progression was observed following adoptive transfer of the *in vitro* expanded Tr1, ApoE-deficient mice immunized with OVA/CFA or mice treated with intranasal HSP60 (44) showed a marked reduction in atherosclerotic plaques size following Tr1 cell infusion (45). Furthermore, a recent study showed the frequency of Tr1 cells, IL-10 and LAG-3 expression by Tr1 cells was lower in patients with coronary artery disease as compared to healthy controls (46). However, there were no observed differences in suppression of Teffs proliferation after incubation with Tregs from patients or healthy subjects (46). Therefore, new studies are urged to better elucidate the role of these subsets of Tregs in CVD and atherosclerosis.

IMMUNOMETABOLISM OF TREGS IN THE CONTEXT OF ATHEROSCLEROSIS

Immunometabolism is an emerging field that investigates the interplay between immunological and metabolic processes. In addition to their exogenous antigen providing role, the contributions of microorganisms to atherogenesis are now beginning to be elucidated. Several factors including environment, diet, medication, genetics, and pathology affect the dynamic composition of the microbiota. Microorganisms produce various metabolites and nutrients such as vitamins and short-chain fatty acids (SCFA) which in turn can influence Treg generation, function, and trafficking (47). Such metabolites can be either systematically disseminated within the bloodstream for example endotoxin LPS; high levels of which is associated with cardiometabolic disorders and inflammation (48), conversely, they can remain in-suit at the site of production acting locally (48).

Butyrate is a SCFA produced by the fermentation of dietary fiber and is highly enriched in the colon (49). SCFAs are known to promote Tregs differentiation via several mechanisms; of particular note via their action as histone deacetylase (HDAC) inhibitors they are able to maintain the acetylation of the FOXP3 promoter at CNS1 and CNS3 which confers increased expression (47). Specifically, in the setting of atherosclerosis, it was demonstrated that gut-colonization of germ-free ApoE mice with strains of bacteria which differed in butyrate production; could affect the progression of atherosclerosis. Data

indicated the presence of bacterial genus *Roseburia,* which is associated with high butyrate production, inversely correlated with atherosclerotic lesion size (49). However, this study failed to find significant differences between Treg populations in either the spleen or para-aortic lymph nodes from mice colonized with the two different communities (49). Further investigation will be required to fully understand the contribution of such factors to human atherosclerosis.

It is not only SCFA which have been associated with atherosclerosis; evidence suggests that certain vitamin intake is beneficial in preventing CVD. Vitamin A, C, E, and K deficiencies are all associated with increased CVD (50). Dietary sources of vitamin A are mainly retinol and retinyl esters from animal origin or, from plant sources provitamin A carotenoids which comprise numerous isomers of β-carotene (51). Vitamin A is metabolized into all-trans retinonic acid (RA); which is important for Treg development in the gut (52). Results in ApoE$^{-/-}$ mice show that a vitamin A deficient diet resulted in an increase in both plasma cholesterol concentration and atherosclerotic lesion size as compared to healthy controls (51). Furthermore, dietary fortification with β-carotene protected against both elevated plasma cholesterol and increased lesion size in mice fed a vitamin A-deficient diet (51). The mechanisms by which β-carotene protected against these adverse outcomes was unclear. However, results in atherosclerotic patients who received 25,000 IU retinyl palmitate per day for 4 months showed an increased expression of FOXP3 in phytohemagglutinin-activated cells as compared to both healthy controls and patients receiving placebo (53), supporting the existence of a link between dietary habits, Tregs, and atherosclerosis.

THE EFFECT OF STATINS ON HUMAN TREGS

Tregs act to prevent atherosclerosis in a range of manners. Although currently no Treg therapies for atherosclerosis exist, some existing treatments have beneficial effects on Tregs. Statins are one of the most widely prescribed treatments for atherosclerosis due to their capacity to reduce cholesterol biosynthesis, it has been reported that these drugs can have other athero-protective effects.

Both atorvastatin and paravastatin attenuate T-cell activation, proliferation, inhibit the secretion of the pro-inflammatory cytokines and enhance secretion of anti-inflammatory cytokines (54). These statins inhibit IFN-γ production, which reduces Th1 activation (54), they also bind to lymphocyte function associated antigen-I (LFA-1) preventing leukocyte adhesion to ECs (54). Both mechanisms could be attributed to the indirect effect statins on Tregs (54). Indeed, both paravastatin and atorvastatin increases Treg numbers, which contributes to down-modulation of IFN-γ producing Th1 cells and reduction on EC activation. Atorvostatin treatment of human cells resulted in increased numbers of CD4$^+$CD25$^+$FOXP3$^+$ Tregs *in vitro,* in addition to enhancing FOXP3$^+$ expression (54). Similar results were reported with the used of pravastatin, which increased the number of CD4$^+$CD25$^+$ cells in humans. Moreover, simvastatin potentiates *ex vivo* Treg expansion.

Therefore, the benefits of statins can be partially attributed to their effects on Tregs. However, no direct analyses of the statins on Treg function and gene expression has been made, nor have how statins affect sub-populations of Tregs in humans been investigated.

THE BIDIRECTIONAL RELATIONSHIP BETWEEN TREGS AND RISK FACTORS

Further to the Treg beneficial effects in atherosclerosis, disease risk factors, such as hyperlipidemia and hypertension, also affect Treg numbers and functions. Indeed, hypercholesterolemia changes plasma membrane dynamics of leukocytes, which supports the proliferation of activated T-cells as well as the size and function of the Treg cell population (55). ApoE$^{-/-}$ mice have reduced numbers of thymic Tregs and express lower levels of CD25 concomitant with an increase in effector T-cell numbers. Moreover, Tregs from these hyperlipidemic mice are less effective at suppressing Teffs *in vitro* as compared to their WT counterparts (56).

The link between hypertension and the adaptive immune system has long been established. There is strong evidence in literature indicating that innervation of the lymphoid organs provide a pathway for direct modulation of blood pressure (57). Additionally, athymic and Rag1 deficient mice do not have increases in blood pressure after treatment with Angiotensin II or deoxycorticosterone acetate (DOCA) as compared to controls (58). The role of Tregs specifically was shown in a hypertensive rat model; Treg depletion resulted in higher blood pressure values and aggravation of cardiac hypertrophy (57). Furthermore, adoptive transfer of FOXP3$^+$ Tregs protected against AngII induced hypertension (57). Accordingly, a recent study developed in our laboratory confirmed such finding and revealed Nox2 deficient Tregs are more potent in inhibiting blood pressure increases and heart fibrosis as compared to WT Tregs (59).

Extensive studies evaluating human Tregs in the context of hypertension are yet to be undertaken. Only one study published in 2018 exists, in which authors reported the down-regulation of Helios$^+$ Tregs in hypertensive patients as compared to their normotensive counterparts (60). Additionally, CD4$^+$ T-cells from hypertensive patients have lower FOXP3 mRNA levels than cells from healthy controls (60). This data might suggest that the hypertensive microenvironment can negatively impact Tregs populations.

Studies using Tregs in setting of both hypertension and hyperlipidemia indicate that, despite Tregs being athero-protective, they are decreased in frequency and functionality in patients suffering from these conditions, which favors the progression to CVD.

IS TREG THERAPY A POSSIBILITY FOR ATHEROSCLEROSIS?

As a result of extensive evidence indicating the beneficial role of Tregs in atherosclerosis, there is an increasing interest regarding the potential use of these cells for immunomodulation. The

use of exogenously expanded and functional Tregs may prove useful in combatting both the defective cell functionality and decreased frequency observed in atherosclerosis. Such therapy has previously been shown to be effective with no significant adverse effect in other diseases including transplantation (61), graph vs. host (61), and autoimmunity in the setting of diabetes melitus (62). The lack of adverse effects is largely attributed to the use of the recipient own cells preventing the elicitation of a detrimental auto-immune response or organ-rejection (61).

Despite such positive observations, prior to the use of exogenously expanded Tregs for treatment of human CVD and atherosclerosis further investigation is needed. A recent study demonstrated that Tregs from mice which had undergone a non-reperfused myocardial infarction exhibited defective capacity to suppress *in vitro* Teff proliferation (63). Such finding highlight that the expansion of the global autologous Treg population may not be an appropriate method to use to halt progression of such diseases. Instead a more tailored approach may be needed; in this regard, our group have published some studies those suggest the use of engineered Tregs, those exhibits a higher suppressive ability (59, 64, 65).

CLINICAL TRIALS

Despite increasing interest in the role of Tregs in atherosclerosis, only a few clinical trials have begun to investigate their significance.

In 2010, a randomized interventional clinical trial by Del Core at Creighton University (NCT01183962) was initiated with the goal of evaluating the potentially beneficial role of oral vitamin D supplementation in patients aged 30–80 with a history of CVD in order to prevent detrimental cardiovascular events. Patients were divided into two groups, one receiving a daily oral dose of 3,000 IU of vitamin D, the other receiving no treatment. The primary endpoint was the analysis of Treg suppressive function, which was expected to improve, independently of cell number. Unfortunately, this study was terminated due to slow enrollment and funding difficulties.

In 2016, Prof. Didier Ducloux at Center Hospitalier de Besançon started the ORLY-Est trial (NCT02843867), an observational prospective study based on the immuno-monitoring of renal transplanted patients for atherosclerotic complications occurring 5 or 10 years' post-operatively. The hypothesis was that by evaluating the percentage of Tregs a prediction could be made about the likelihood of atherosclerotic complications occurring. A value under the median would be associated with a higher incidence of atherosclerotic complications by 5%. This observational study is expected to lead to a second trial (ORLY-IS) to test the effect of Treg expansion on the incidence of detrimental atherosclerotic events after transplantation.

In 2017, the group led by Johann Motsch at University Hospital Heidelberg, designed the LeukoCAPE-2 trial (NCT03105427), an observational case-only study to evaluate the use of Tregs to predict the cardiovascular risk in patients with known CVD undergoing major non-cardiological surgery, and those post cardiovascular surgery. Overall, 233 patients were enrolled, and blood was drawn at pre-defined time points up to 3 days post-operatively. Clinical follow up for cardiovascular events was carried for 30 days post-surgery. The primary outcome was the occurrence of cardiac death and/or MI and/or mL and/or myocardial injury after non-cardiac surgery (MINS) and/or embolic stroke and/or thrombotic stroke. To date, the trial is completed, but results are not yet published.

In 2019, the group led by Prof. Hongwei at Beijing Friendship Hospital started an observational, prospective trial (NCT03939338) which aims to evaluate whether the combination of both Treg frequency and cardiac magnetic resonance imaging (CMR) can be used to predict the severity of reperfusion injury following MI. The study is expected to be complete by 2021.

IMMUNOLOGICAL TARGETS WITHIN ATHEROSCLEROSIS

Several studies using animal models have investigated the potential of producing preventative vaccines for atherosclerosis. Analysis of mRNA from ApoE$^{-/-}$ mice indicates T-cells within atherosclerotic lesions show the preferential expression of a limited number of TCR-variable gene segments suggesting that a limited set of antigens are responsible for the specific T-cell response present in atherosclerosis (66). Most of the identified antigens present in atherosclerosis are generated via the modification of self-molecules; previous studies in mice have investigated the potential of some of these antigens as candidate for the production of vaccines.

OxLDL has been investigated as a candidate antigen, the generation of mucosal tolerance against oxLDL was achieved via its oral administration in LDLR$^{-/-}$ mice prior to the onset of atherosclerosis. Oral administration attenuated both the initiation and progression of the disease. Furthermore, increased numbers of Tregs specific for oxLDL were observed in both the spleen and lymph nodes following immunization (39). ApoB100 is the peptide component of LDL and is displayed on the surface of APCs via MHCII molecules following proteolytic processing (9). Continuous sub-cutaneous infusion of ApoB100 derived peptides in ApoE$^{-/-}$ mice resulted in reduced atherosclerotic plaque development, in addition to inhibiting the progression of previously established disease and promoting features of plaques healing such as increased collagen content, and decreased T-cell infiltration (67). Evidence indicates that the mucosal administration of the ApoB100 antigen induces antigen-specific tolerance through the generation of several Treg subsets which could be responsible for the observed athero-protective effects.

HSPs have also been found to act as antigens in atherosclerosis (40). HSP60-specific T-cells are mainly Th1 and thus have a proatherogenic phenotype and produce cytokines such as IFNγ and IL-12. Studies using LDLR$^{-/-}$ mice have shown that induction of oral tolerance to HSP60 results in attenuated atherosclerosis which is attributed to an increased CD4^{+}CD25^{+}FOXP3^{+} Tregs population in both lymphoid

organs and the atherosclerotic lesion. This is accompanied by an increase in HSP60-specific TGFβ and IL-10 production in the mesenteric lymph node cells (40).

These findings in mice indicate several potentially good candidate antigens for the generation of a targeted vaccine. In addition to the aforementioned antigens, there are many others associated with atherosclerosis including collagen, fibrinogen, advance glycation-end products (AGE), (LP(a)), lipoprotein-lipase (LPL), and microbial antigens (9) which have not been explored in the context of targeted therapeutics, and so their potential in the generation of either a preventative vaccine or potentially antigen-specific Tregs for uses as therapeutic treatment in atherosclerosis remains unknown.

DISCUSSION

Despite the increasing global burden of patients with atherosclerosis, a curative therapy is still to be found. Symptom and lifestyle management can act to slow the disease progression but ultimately it will not be totally halted due to its association with aging and vessels inflammation.

Tregs have been closely associated with atherosclerosis in both animal models and humans, with their presence and their mechanisms of action shown to be atheroprotective. Despite this evidence, there has been little investigation into the potential of Treg therapy. The few trials focusing on CVD patients and Tregs have tended to monitor Treg number, function and subtype for potential use as biomarkers for disease severity. One trial did try to utilize Vitamin D to enhance endogenous Treg populations, however this is far from mimetic a cellular therapy involving infusion of exogenously expanded autologous Tregs. As a result, many questions remain surrounding the potential use of Tregs in atherosclerosis and other chronic inflammatory diseases involving the cardiovascular system.

The production of antigen-specific Tregs is an attractive option. Indeed, such technologies are being utilized in pre-clinical models of transplantation (64, 68). However, the suppressive efficiency, stability and migratory capacity of genetically engineered Tregs need further evaluation before they can be used in the clinic.

In summary, Tregs present very promising targets with a great deal of potential. However, as a new and emerging field, it is important to carefully find a safe and efficient method for such a cellular therapy. Once achieved, Treg therapy could potentially become a viable treatment option in the battle against atherosclerosis and CVD.

AUTHOR CONTRIBUTIONS

GG participated in manuscript writing. CA and SCT contributed to manuscript writing, figure development, and manuscript editing. GL and CS contributed to manuscript editing.

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REVIEW
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Metabolic Pathways Involved in Regulatory T Cell Functionality

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Regulatory T cells (Treg) are well-known for their immune regulatory potential and are essential for maintaining immune homeostasis. The rationale of Treg-based immunotherapy for treating autoimmunity and transplant rejection is to tip the immune balance of effector T cell-mediated immune activation and Treg-mediated immune inhibition in favor of Treg cells, either through endogenous Treg expansion strategies or adoptive transfer of *ex vivo* expanded Treg. Compelling evidence indicates that Treg show properties of phenotypic heterogeneity and instability, which has caused considerable debate in the field regarding their correct use. Consequently, for further optimization of Treg-based immunotherapy, it is vital to further our understanding of Treg proliferative, migratory, and suppressive behavior. It is increasingly appreciated that the functional profile of immune cells is highly dependent on their metabolic state. Although the metabolic profiles of effector T cells are progressively understood, little is known on Treg in this respect. The objective of this review is to outline the current knowledge of human Treg metabolic profiles associated with the regulation of Treg functionality. As such information on human Treg is still limited, where information was lacking, we included insightful findings from mouse studies. To assess the available evidence on metabolic pathways involved in Treg functionality, PubMed, and Embase were searched for articles in English indexed before April 28th, 2019 using "regulatory T lymphocyte," "cell metabolism," "cell proliferation," "migration," "suppressor function," and related search terms. Removal of duplicates and search of the references was performed manually. We discerned that while glycolysis fuels the biosynthetic and bioenergetic needs necessary for proliferation and migration of human Treg, suppressive capacity is mainly maintained by oxidative metabolism. Based on the knowledge of metabolic differences between Treg and non-Treg cells, we additionally discuss and propose ways of how human Treg metabolism could be exploited for the betterment of tolerance-inducing therapies.

Keywords: metabolism, human Treg cells, FOXP3, proliferation, migration, suppressive function, tolerance-inducing therapies

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INTRODUCTION

To safeguard ourselves, our immune system is equipped with a series of defense systems to recognize and respond to *non-self*-structures. Although essential for fighting off infections and preventing cancers from arising, destructive immune responses pose a considerable challenge in autoinflammation and transplantation. Currently available immunosuppressants help to

control destructive immune responses. However, management of side-effects of lifelong immunosuppression, including cancer development and reduced survival, remain major problems (1). For this reason, an increasing amount of interest is directed toward the natural specific regulatory mechanism of the immune system. A better understanding of these mechanisms holds the key to the development of novel immunomodulatory therapies (2).

One approach the immune system employs to induce *self*-tolerance is via regulatory T cells (Treg). Treg modulate the immune system both specifically and aspecifically via inhibition of dendritic cell function and maturation, secretion of anti-inflammatory cytokines, and suppression of induction and proliferation of antigen-specific effector T cells (Teff) (3), depicted in **Figure 1**. As reviewed previously (4), human Treg are characterized by expression of the transcription factor Forkhead box p3 (FOXP3) and the combination of cell surface markers $CD4^+$, $CD25^+$, and $CD127^{low/-}$. FOXP3 is the most reliable cell marker for Treg, although it is also transiently expressed by activated effector T cells. Nevertheless, FOXP3 expression is essential for the maturation and function of Treg. Suppressive capacity of Treg is commonly associated with the amount of FOXP3 expression. Also strength, binding and expression of the T cell receptor (TCR), capacity to produce anti-inflammatory cytokines like interleukin-10 (IL-10) and IL-35, and expression of suppressive cell membrane molecules like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), CD39, and CD73 are fundamental in this (3), as depicted in **Figure 1**. Of note, expression levels from some of these molecules have been linked to metabolism. Following Treg activation, mTOR signaling is upregulated which induces lipid synthesis, mevalonate metabolism and mitochondrial function (5). CDTLA-4 and PD-1 have both been described to block glycolysis whereby PD-1 signaling also promotes lipolysis and fatty acid oxidation (6). CD39, which converts adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into adenosine monophosphate (AMP), and CD73, which subsequently converts AMP to adenosine, abrogates ATP-effects such as P2 receptor-mediated cell toxicity and ATP-driven maturation of dendritic cells.

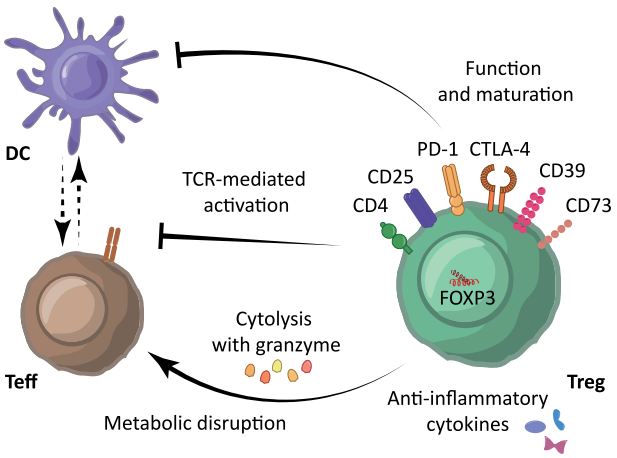

FIGURE 1 | Immunosuppressive mechanisms underlying Treg-mediated immune suppression. Treg are characterized by expression of the cell surface markers $CD4^+$, $CD25^{high}$ and $CD127^{low/-}$, and transcription factor FOXP3. Treg modulate the immune system using their suppressive molecules PD-1, CTLA-4, CD39, and various surface receptors through inhibition of dendritic cell (DC) function and maturation, through the secretion of anti-inflammatory cytokines such as IL-10, TGF-β and IL-35, and/or through direct inhibition of Teff via induction of cytolysis using granzyme and metabolic disruption. Moreover, Treg can reduce Teff activation by limiting TCR-ligand binding.

Human Treg are currently intensively studied for the induction of immunotolerance both in transplantation and autoimmunity (7). Although these studies have substantially advanced our knowledge on Treg, important issues on the optimization of these therapies regarding Treg expansion, homing and stability of immunoregulatory function remain to be resolved. Here, we asked ourselves whether employing knowledge on cell metabolism can be of benefit for the advancement of Treg therapy.

Cell metabolism was shown to be a fundamental determinant of immunological cell fate and function (8). Most resting immune cells are relatively metabolic inactive. However, during activation, immune cells increase their metabolic requirements and switch their metabolic programs to accommodate the increased demand for energy and biosynthesis (8). Although glycolysis is a rapid method for energy production and it supports other metabolic pathways by producing nicotinamide adenine dinucleotide (NADH), it is a relatively inefficient energy

Abbreviations: 2-DG, 2-deoxy-D-glucose; 3-HAA, 3-hydroxy anthranilic acid; A2A, Adenosine 2A receptor; Acetyl-CoA, Acetyl coenzyme A; ADP, Adenosine diphosphate; AHR, Aryl hydrocarbon receptor; AMP, Adenosine monophosphate; AMPK, AMP-activated protein kinase; ARG1, Arginase 1; ASM, Acid sphingomyelinase; ATP, Adenosine triphosphate; CCR7, C-C chemokine receptor 7; CD62L, Leukocyte adhesion molecule L-selectin; CPT1, Carnitine palmitoyltransferase 1; CPT2, Carnitine palmitoyltransferase 2; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; DEPTOR, DEP domain-containing mTOR-interacting protein; EZH2, Enhancer of zeste homolog 2; FAO, Fatty acid oxidation; Foxo, Forkhead box protein O; FOXP3, Forkhead box p3; GCK, Glucokinase; HIF-1α, Hypoxia-inducible factor 1α; HK, Hexokinase; HMGR, HMG-CoA reductase; ICOS, Inducible co-stimulator; IDO, Indoleamine-pyrrole 2,3-dioxygenase; INSR, Insulin receptor; IRF4, Interferon regulatory factor 4; LDH, Lactate dehydrogenase; LFA-1, Lymphocyte function-association 1; MAPK, Mitogen-activated protein kinase; Mef2, Myocyte enhancer factor 2; mTOR, Mammalian target of rapamycin; mTORC1, Mammalian target of rapamycin complex 1; mTORC2, Mammalian target of rapamycin complex 2; NAD^+, Oxidized nicotinamide adenine dinucleotide; NADH, Reduced nicotinamide adenine dinucleotide; $NADP^+$, Oxidized nicotinamide adenine dinucleotide phosphate; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; Ndfip1, Nedd4-family E3 ubiquitin ligases; NFAT, Nuclear factor of activated T-cells; OXPHOS, Oxidative phosphorylation; PD-1, Programmed cell death protein 1; PDH, Pyruvate dehydrogenase; PDHK1, Pyruvate dehydrogenase kinase 1; PDK1, Phosphoinositide-dependent kinase 1; PI3K, Phosphatidylinositol 3-kinase; PPARγ, Peroxisome proliferator-activated receptors γ; PPP, Pentose phosphate pathway; PTEN, Phosphatase and tensin homolog; pTreg, Periphery-inducible regulatory T cell; RAPTOR, Regulatory-associated protein of mTOR; RICTOR, Rapamycin-insensitive companion of mTOR; ROS, Reactive oxygen species; S1PR1, Sphingosine-1-phosphate receptor 1; SIRT1, Silent mating type information regulation 2 homolog; TCA cycle, Tricarboxylic acid cycle, or citric acid cycle, or Krebs cycle; TCR, T cell receptor; Teff, Effector T cell; TGF-β, Transforming growth factor beta; Treg, Regulatory T cell; tTreg, Thymic-derived regulatory T cell; VAT, visceral adipose tissue.

source compared to the mitochondrial oxidative metabolism of oxidative phosphorylation (OXPHOS) (**Figure 2**). In glycolysis, glucose is converted in the cytoplasm into glucose-6-phosphate by hexokinase (HK) or glucokinase and subsequently converted into pyruvate or shuttled into the pentose phosphate pathway (PPP). During the PPP, nicotinamide adenine dinucleotide phosphate (NADPH), a precursor for the synthesis of nucleotides and amino acids, is produced. Pyruvate resulting from glycolysis can either be converted into lactate by lactate dehydrogenase (LDH) or converted into acetyl-CoA to enter the tricarboxylic acid (TCA) cycle. The TCA cycle, also known as the citric acid cycle or the Krebs cycle, is the primary metabolic pathway of quiescent or non-proliferating cells and a highly efficient source of energy. Besides acetyl-CoA from pyruvate, the TCA cycle is also fueled by acetyl-CoA produced during fatty acid oxidation, α-ketoglutarate following glutaminolysis, and by the acids citrate and succinate. These can subsequently proceed into OXPHOS, which is a less rapid but more energy-efficient pathway. Acetyl-CoA from glycolysis and fatty acid oxidation (FAO) can also support the mevalonate pathway. OXPHOS follows fatty acid oxidation and the TCA cycle can be followed by fatty acid synthesis. Both glycolysis and fatty acid oxidation support the mevalonate pathway via acetyl-CoA.

Generally, glycolysis tends to support the function of pro-inflammatory cells, such as Teff and M1 macrophages, while OXPHOS and FAO tend to be used by anti-inflammatory cells, such as M2 macrophages, memory $CD8^+$ Teff and Treg. Resting T cells mostly utilize a catabolic oxidative metabolism of glucose, lipids, and amino acids, whereas, upon activation T cells upregulate their glucose and amino acid transporters and increase their glycolytic flux to fuel the enhanced glycolysis process, thus supporting their proliferation and inflammatory functions. While the metabolic requirements for conventional T cells are increasingly understood, those important for Treg have been less well-defined. Mostly, Treg are assumed to rely more on the TCA cycle and OXPHOS than on glycolysis, leaving the complex interplay between metabolism and functional state underexposed. Thus, a better insight into Treg metabolism and its relevance for tolerance-inducing therapies could benefit further improvement of tolerance-inducing therapies. The characterized metabolic pathways involved in Teff and Treg are summarized in **Table 1**. In this review, we will detail the contribution of these metabolic pathways to Treg functionalities with the focus on cell proliferation, migration and suppressive capacity. Furthermore, we will discuss exploiting Treg metabolism for tolerance-inducing therapies. Although the main focus of this review is on human Treg, we also included insightful findings from mouse studies in cases where human information was lacking.

METHODS

To assess available evidence on metabolic pathways involved in human Treg functionality, PubMed, and Embase were searched for articles indexed in English before April 28th, 2019 for "regulatory T lymphocyte," "cell metabolism," "cell proliferation," "migration," "suppressor function," and related search items (**Supplementary Table S1**). Where studies on human Treg metabolic profiles were lacking, we included insightful findings from mouse studies. Removal of duplicates and search of references was performed manually.

METABOLIC PATHWAYS LINKED TO Treg BEHAVIOR AND FUNCTION

Proliferative Behavior and Development of Treg

To manage the accompanying increased demand for energy and biosynthesis during cell growth, the engagement of specific metabolic pathways is essential. Proliferative cells have typically high glycolytic rates and employ the glycolysis-diverting pathways PPP and serine biosynthesis pathway for energy while utilizing glutamine as fuel for the synthesis of biomass (**Figure 2**). In cancer cells, this switch is referred to as the Warburg effect (9). A similar phenotype has been described for T cells (10).

Glucose is the major source for generating ATP. Glucose can be broken down via either the glycolysis process, i.e., in the cytoplasm converting glucose to pyruvate which is further diverted to lactate by LDH, or the TCA cycle when pyruvate dehydrogenase (PDH) converted pyruvate to acetyl-CoA. Upon activation, T cells preferentially use glycolysis for producing ATP to meet their rapid expansion.

In contrast to Teff cells, which mainly use glycolysis following stimulation, memory T cells, as well as Treg, have a quite different metabolic state, since they largely rely on the FAO for cell persistence and function. Pro-growth signaling pathways, including phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), as well as mammalian target of rapamycin (mTOR) promote glycolysis in Treg (11). Glucose uptake in Treg is mostly mediated by differential expression of the glucose transporter GLUT1. Interestingly, Glut1 expression is higher on activated murine induced Treg (iTreg) than proliferated tTreg, and Glut1 enhances Treg proliferation but inhibits their suppressive function. Notably, GLUT1 expression is reduced by the Treg transcription factor FOXP3. tTreg from mice with transgenic T cell-specific expression of Glut1 showed impaired lineage stability as they had decreased expression of CD25/EZH2 and secreted IFNγ (11). Inhibition of mTOR in Treg greatly diminishes glucose uptake, but it helps to maintain a stable Treg phenotype and high suppressive capacity (12). Depletion of glucose is detrimental for Treg proliferation since Treg mitochondrial oxidize lipid as well as glycolysis derived pyruvate at high speed (13). Of interest, human carriers of a loss-of-function glucokinase (GCK) regulatory protein gene that leads to an enhanced GCK activity, have reduced circulating Treg numbers (14), indicating that either Treg proliferation is impaired or the Treg have migrated to tissue sites. Moreover, evidence showed that inhibition of glycolysis could steer T cells toward Treg differentiation (15), and this happens by increasing FOXP3 expression via the inhibition of mTOR-mediated induction of the transcription factor hypoxia-inducible factor-1α (HIF-1α) (15, 16).

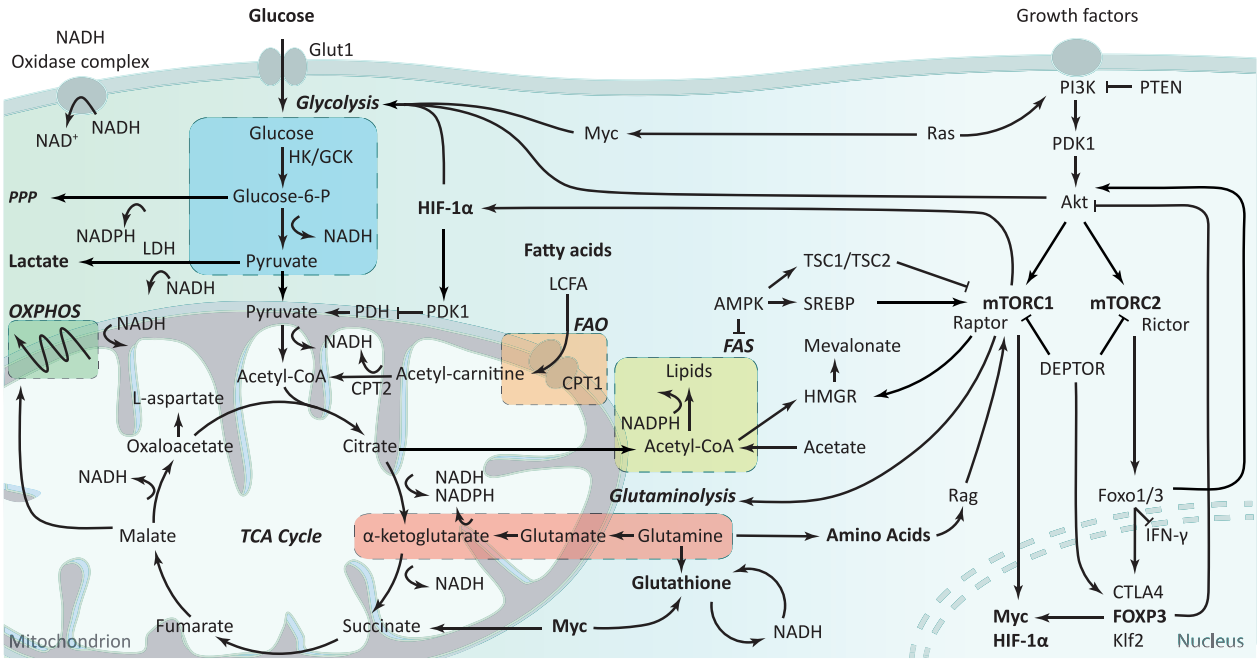

FIGURE 2 | Overview of cellular metabolism in T cells. The first step of glycolysis is the conversion of glucose into glucose-6-phosphate by hexokinases (HK) or glucokinase (GCK). During glycolysis, glucose can be shuttled away toward the pentose phosphate pathway (PPP), or stay in glycolysis resulting in its conversion into pyruvate. Pyruvate can either be converted to lactate by lactate dehydrogenase (LDH), using the nicotinamide adenine dinucleotide (NADH) produced during glycolysis, or it can be converted into acetyl-CoA and enter the tricarboxylic acid (TCA) cycle. The TCA cycle can also be fueled by acetyl-CoA produced during FAO and α-ketoglutarate following glutaminolysis. Subsequently, the TCA cycle fuels OXPHOS and FAS. NADH and reduced nicotinamide adenine dinucleotide phosphate (NADPH) are produced during the conversion of various metabolites and play an important role in proton homeostasis and ROS production via the $NADP^+$ complex and glutathione metabolism. Although all these metabolic pathways are interlinked, cells can regulate their isolated activity and compensate for alterations in the different pathways. The PI3K-Akt-mTOR pathway plays a role in metabolic regulation through various routes. Glycolysis is stimulated via Akt and HIF-1α while fatty acid synthesis and amino acid synthesis are impacted by and impact mTORC1. The PI3K-Akt pathway also modulates FOXP3 expression, which itself impact metabolism via Myc and Akt signaling. OXPHOS, oxidative phosphorylation; FAO, fatty acid oxidation; FAS, fatty acid synthesis.

Actively proliferating T cells change from a catabolic metabolism to an anabolic metabolism, in which the fatty acids and amino acids are shunted away from the TCA cycle into membrane and protein synthesis, respectively (**Figure 2**). HIF-1α plays an important role in the decision to commit pyruvate to lactate for glycolysis or to acetyl-CoA for entering the TCA cycle. HIF-1α induces the expression of glycolytic genes and increases LDH enzyme activity, thus enhancing the conversion of pyruvate to lactate and leading to a glycolysis-shift. It also actively represses mitochondrial function via the induction of pyruvate dehydrogenase kinase 1 (PDK1) (17, 18). PDK1 inhibits PDH and thereby reduces the mitochondrial oxidization of glycolysis-derived pyruvate via the transformation of pyruvate to acetyl-CoA, which acts as the intramitochondrial starting point of OXOPHOS (**Figure 2**). Inhibition of PDK1 increases Treg numbers in mice (19), which is independent of PDK itself, but through the production of reactive oxygen species (ROS), potentially due to the increased capacity of Treg to scavenge ROS away compared to other T cell subsets (13). Both nTreg and iTreg have higher mitochondrial mass and higher ROS production which are correlated with increased FOXP3 expression (13). ROS is produced following the TCA cycle during subsequent OXPHOS. Although ROS plays important roles in cell signaling and homeostasis, inhibition of OXPHOS does not affect Treg differentiation (20).

Although Treg mainly use oxidative metabolism for growing, Treg proliferation is dependent on an oscillatory switch of glycolysis (21). In mice, both glycolysis and OXOPHOS are involved in the generation of iTreg as well as the growth of Teff cells (13). Freshly isolated human Treg show a high metabolic state including increased mTOR pathway, high amount of phospho-STAT5, and hyporesponsiveness *in vitro*. Interestingly, transient inhibition of mTOR, before TCR stimulation, promotes TCR-induced Treg proliferation, while later (60–72 h after stimulation) actively proliferating Treg cells displayed high mTOR activity (21). It seems that Treg proliferation is closely associated with the dynamic changes in mTOR activity which is influenced by the composition of nutrients within the extracellular milieu.

Treg exhibit increased FAO and enhanced expression of genes involved in FAO during proliferation (19, 22). FAO is a multistep process in which the long-chain fatty acids are first conjugated to carnitine via the rate-limiting enzyme carnitine palmitoyltransferase 1 (CPT1). These are subsequently

TABLE 1 | Characterized metabolic pathways involved in human effector and regulatory T cells.

	Effector T cells (Teff)	Regulatory T cells (Treg)
Metabolic pathways in quiescent cells	Catabolic oxidative metabolism of glucose, lipids, and amino acids	Fatty acids oxidation (FAO) OXPHOS
Energy sources upon activation	Aerobic glycolysis Mitochondrial respiration	Mitochondrial oxidation of lipids, and pyruvate *in vitro* and *in vivo* Highly glycolytic *in vivo*
Glucose transporter	High GLUT1	Low GLUT1
Glutaminolysis	Critical for Th1/Th17 differentiation	Inhibits iTreg generation
Amino acids metabolite	Crucial for Teff proliferation and cell survival	Promote iTreg differentiation Crucial for Treg function
Mitochondrial mass and ROS	Low	High
Fatty acids	Increase glycolytic flux Promotes Th1/Th17 differentiation	SCFAs promote Treg differentiation and function
PPARγ	Inhibits Th17 differentiation	Maintenance and accumulation of Treg in adipose tissue
PI3k/Akt/mTOR signals	High (upon activation)	Low (upon activation)
Basal level of mTORC1 activation	Low	High
Signals via AMPK	Low Pro-survival function of AMPK	High Promotes Treg generation
Signals via Myc and HIF-1α	High (upon activation) Promote Th17 differentiation	Low (upon activation) Impairs iTreg generation and Treg lineage stability

AMPK, AMP-activated protein kinase; mTORC1, mTOR complex 1; PPAR, peroxisome proliferator-activated receptors; SCFA, short-chain fatty acids.

shuttled to the mitochondrion and converted to acyl-CoA by carnitine palmitoyltransferase 2 (CTP2). This acyl-CoA is further degenerated by β-oxidation to produce acetyl-CoA, which then enters the TCA cycle. CPT1 inhibitor etomoxir inhibits Treg, but not Teff differentiation and proliferation, demonstrating a selective dependency of Treg on FAO. Both murine tTreg and iTreg show higher mitochondrial mass and increased ROS production as compared to Teff or non-T cells (13). Upstream, activated AMPK release the inhibition of CPT1 thus allowing the transport of long-chain fatty acids to the mitochondria for subsequent FAO and ATP generation. Of interest, Treg show high levels of activated AMPK and treatment with metformin, an indirect activator of AMPK, can decreases total T cell numbers, while increasing the percentage and number of Treg (23). Leptin, a cytokine-like hormone stimulating FAO and glucose uptake, constrains the proliferation of Treg through AMPK and mTOR activation (24). Transient inhibition of the leptin-mTOR pathway promotes TCR-induced proliferation in Treg, while an intact mTOR pathway is needed to sustain Treg proliferation (21). This seeming contradiction might be explained by a need for a lower metabolic rate to enter the cell cycle and start proliferation in Treg. In fact, it is shown that *in vitro* proliferation of human Treg requires both glycolysis and FAO (25).

The dependence of Treg on FAO is more evident in tissue-resident Treg, such as visceral adipose tissue (VAT) Treg and intestinal mucosa Treg. VAT Treg are specifically recruited to adipose tissue to suppress the local inflammatory process (26). VAT Treg uniquely express PPARγ (peroxisome proliferator-activated receptors γ), which is crucial in peroxisomal-mediated β-oxidation of FAO, and show a high expression of CD36, a receptor that facilitates the import of fatty acids. Like other Treg, VAT Treg also express leptin-receptors. Leptin binding to its receptor would lead to high activation of mTOR, which affects Treg proliferation. In mice, it has been shown that adipose tissue of obese mice contains high levels of leptin, associated with decreased numbers of Treg, as opposed to lean mice (27). Another example of the effect of anatomical location on Treg metabolism comes from mucosal Treg. The intestinal environment is known to be rich in short-chain fatty acids, such as propionate and butyrate that are generated from the fermentation of dietary fiber. The short-chain fatty acids have been described to influence Treg numbers *in vivo*. Butyrate enhances histone H3 acetylation in the promoter region of FOXP3 and thereby boosts extrathymic Treg formation (28). Additionally, butyrate appears to mediate Treg differentiation by engagement with gut-epithelial cells that subsequently produce IL-10 and a range of metabolites, including retinoic acid, which leads to the production of inducible Treg (29). Propionate, which is also capable of increasing histone acetylation, also promotes Treg generation, while acetate cannot increase histone acetylation and promote Treg generation (30).

Amino acids are used as substrates in various metabolic pathways. Most noteworthy, glutamic acid derivates such as glutamine and glutamate fuel the TCA cycle and scavenge ROS. Correspondingly, the availability and metabolism of amino acids play a decisive role in iTreg generation. Although Treg have lager reserve pools of reduced and oxidized glutathione to cope with higher levels of ROS in Treg, deprivation of glutamine steers Treg generation even in conditions favoring other T cell subsets, whereas supplementation with glutamine supports differentiation toward Th1 (31). Similarly, depletion of arginine and tryptophan *in vitro* cell cultures stimulates Treg generation (32). Interestingly, the byproduct of tryptophan catabolism seems to benefit the iTreg generation since the administration

of tryptophan enhances the number of Treg (33). Moreover, Treg are known to highly express amino acids catabolizing enzymes arginase 1 (ARG1) that is responsible for the depletion of extracellular L-arginine thus limiting T cell proliferation, suggesting that Treg could sense the concentration of certain amino acids and/or their byproducts in the local milieu and thereby adjusting the suppressive function properly.

In summary, overall evidence suggests that, compared to other T cell subsets, Treg appear to demonstrate a selective dependency on FAO during proliferation, while being less dependent on glycolysis (**Figure 3A**). As compared to Teff, in general, Treg show a higher level of lipid oxidization as well as the mitochondrial oxidization of glucose. Glucose is required for Treg cell growth and is a key requirement at the early phase of iTreg generation. Activated iTreg expressed highest GLUT1 transporter, although it is still quite low as compared to non-Treg cells, than proliferated tTreg. Blocking of glycolysis seems to promote the generation of iTreg through mTOR-mediated regulation of HIF-1α, which in turn prevents the mitochondrial oxidation of glucose thus leading to a glycolysis shift. Like non-Treg cells, Treg adapt to their environmental nutrient and oxygen status via the opposing actions of mTOR, AMPK, and HIF-1α. Conditions that decrease mTOR activation permit FOXP3 expression, which in turn re-programs T cells to enhance the expression of genes involved in FAO. The oscillatory changes of leptin-mTOR pathways (early downregulation of mTOR activity followed by a full activation of mTOR pathway during Treg expansion) seem to set the threshold for Treg proliferation. FAO is especially crucial to keep the optimal number of tissue-resident Treg, and the metabolism of amino acids is crucial for the generation of iTreg.

Migratory Behavior of Treg

Proper orchestration of immune responses and suppression thereof requires appropriate control of Treg migration within both lymphoid and non-lymphoid organs. As reviewed by Chow et al., mechanisms of Treg migration are diverse and differ according to their developmental stage, role and tissue target (34). To be able to regulate immune responses when and where needed, tight regulation of expression of adhesion molecules and chemokines receptors according to the developmental stage and the microenvironment of the Treg is essential. Although migration is likely the most energy-consuming cellular activity, the metabolic demands for Treg locomotion are poorly investigated.

Like most migratory cells, Treg engage in glycolysis to meet their bioenergetic needs for migration. Treg motility can be inhibited by depletion of glucose from the culture medium, inhibition of glucose uptake or inhibition of glycolysis. To support their increased glucose consumption, Treg upregulate their insulin receptor (INSR) (35). Kishore et al. have demonstrated that Treg migration requires GCK activation for the conversion of glucose in the activation of glycolysis (14) (also see **Figure 2**). GCK promotes migration following pro-migratory and pro-glycolytic stimuli via PI3K-mTORC2 by cytoskeletal rearrangements and by associating with the cytoskeleton component actin. GCK contribution to human Treg migration was observed in human carriers of a loss-of-function polymorphism in the GCKR gene (C to T, P446L), which lead to an increased GCK activity. 446L-GCKR Treg displayed increased chemokine-induced motility compared to WT-GCKR Treg, although the suppressive ability and phenotype did not significantly differ from WT-GCKR Treg (14).

Treg migration is also regulated by glycolytic feedback control through PI3K-Akt pathways (36). Recent investigations of Finlay et al. have established that activation of Akt downregulates the expression of leukocyte adhesion molecule L-selectin (CD62L), chemokine receptors C-C chemokine receptor 7 (CCR7), and Sphingosine-1-phosphate receptor 1 (S1PR1) by control of Foxo1 and Foxo3 (37). Therefore, Akt activation could result in failure of leukocyte-homing to secondary lymphoid organs and stimulate migration to peripheral tissues in humans (38). Exposure of Treg to the mTOR inhibitor rapamycin suppresses upregulation of both α4β7 and CCR9, suggesting that the mechanism is mTOR-dependent. Strikingly, rapamycin-insensitive companion of mTOR (RICTOR) or (mTORC2)-deficient Treg have unaltered ability to express CCR9, while RAPTOR(mTORC1)-deficient Treg are unable to upregulate CCR9, suggesting the selective participation of mTORC1 in the regulation of Treg motility (39). Loss of phosphatase and tensin homolog (PTEN) also impacts migration by lowering CD62L and CCR7 expression (54). This is suggested to be mediated by master kinase phosphoinositide-dependent kinase-1 (PDK1) signaling, which has an important role in the signaling pathways activated by several growth factors and hormones, including insulin signaling. To retain glycolytic flux, pyruvate is often converted into lactate (**Figure 2**). Extracellular sodium-lactate and lactic acid have been described to entrap $CD4^+$ and $CD8^+$ T cells at sites of infection by repressing their migratory capacity (55). Lactate mediated inhibition of motility both *in vitro* and *in vivo* appears to be caused by interference with glycolysis. This selective control of motility is mediated by the specific monocarboxylate transporter Slc5a12, which amongst others transports lactate and pyruvate across the cell membrane (55). Glycolysis is otherwise activated upon engagement of the chemokine receptor CXCR3 with its ligand CXCL10, stimulating lymphocyte tissue infiltration.

There is limited to no sufficient empirical evidence for metabolic control of Treg migration through the TCA cycle and OXPHOS. Neither engagement of a central mediator of T cell migration, lymphocyte function-association 1 (LFA-1), nor inhibition of fatty acid oxidation impacts Treg migration (14). However, the lipid-activated S1PR1 was reported to induce selective activation of the Akt-mTOR kinase pathway in Treg migration from lymphoid organs to blood (40). Interestingly, although Treg depend similarly on S1PR1 as Teff, S1PR1 drives Treg accumulation in tumors, but not $CD8^+$ T cells (41). It is known that the tumor microenvironment is enriched with indoleamine-pyrrole 2,3-dioxygenase (IDO), which metabolizes tryptophan to kynurenine, an endogenous ligand for the aryl hydrocarbon receptor (AHR). IDO could reduce local tryptophan availability in the proximity of Treg thus contributing to their motility (42).

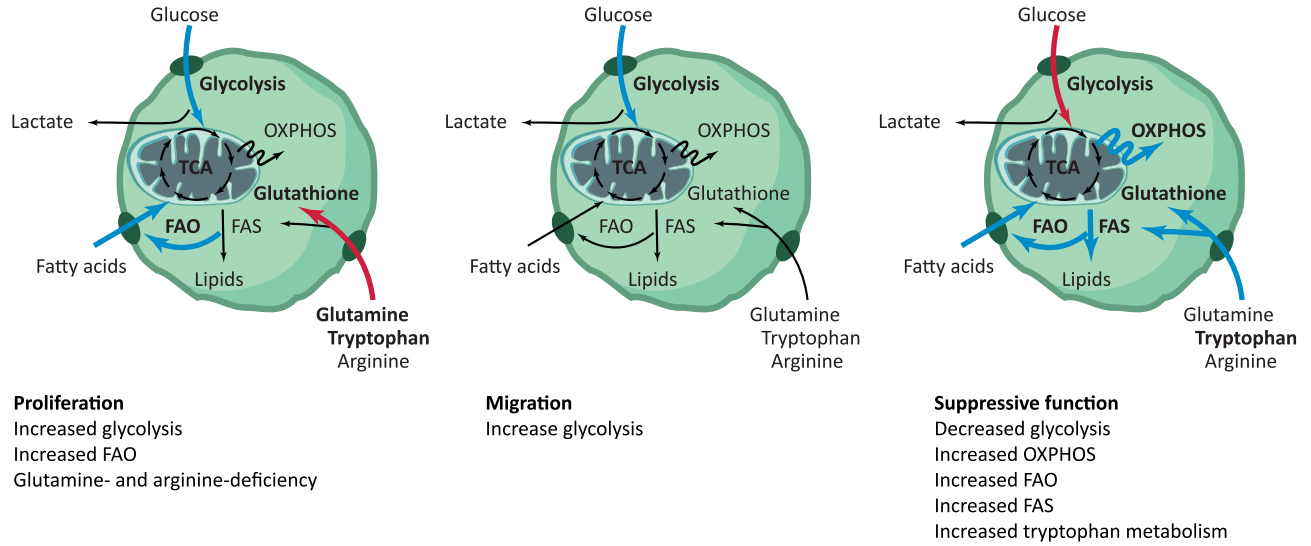

FIGURE 3 | Summary of metabolic pathways involved in distinct Treg functionalities. Treg have distinct metabolic phenotypes throughout their different phases, although many pathways remain to be elucidated. **(A)** During proliferation, Treg have increased glycolysis and FAO. Deficiency of glutamine and tryptophan steers T cells toward Treg differentiation (10–31). **(B)** To support the increased need for energy during migration, Treg increase their glycolytic flux. Metabolic shifts in other pathways have not been described for Treg migration (15, 16, 33–39). **(C)** Treg show decreased glycolysis and increased OXPHOS, FAO, FAS and tryptophan metabolism during their phase of suppressive function. No relevance for the TCA cycle has been reported (6, 10, 14–17, 21, 25, 26, 29, 31, 40–53). The blue and red arrows are indicative for increased or decreased activity of the specific pathway in the functional phenotype of Treg, respectively. FAO, fatty acid oxidation; FAS, fatty acid synthesis; TCA, tricarboxylic acid; OXPHOS, oxidative phosphorylation.

Summarizing, glycolysis is essential to support the bioenergetic needs of Treg migration, like in most migratory cells. mTORC2 plays a non-redundant role in the regulation of Treg motility, probably through regulating the PI3K-Akt pathway, as well as GCK kinase activity to mediate cytoskeleton reorganization (**Figure 3B**).

Suppressive Function and Stability of Treg

To exert their suppressive function, Treg stability, and therefore stable FOXP3 expression is imperative. The stability of Treg is currently the topic of many studies in the Treg field (4). Importantly, besides the loss of suppressive function, Treg can differentiate into proinflammatory cytokine-producing cells (also named exTreg) under specific microenvironmental cues, and induce detrimental immune responses, posing a threat for adoptive Treg therapies. Several transcriptional programs are involved for activated Treg further differentiation into a suppressive effect state. For instance, the transcription factor interferon regulatory factor 4 (IRF4) is essential for mucosal Treg suppressive function and stability. TCR-dependent signals activate mTOR which in turn promotes the expression of IRF4, GATA3 as well as upstream regulators of glycolysis pathway like HK2, Myc, and Foxo (56). Mitochondrial metabolism is highly induced in an mTOR dependent manner during Treg activation since Treg specific depletion of mitochondrial transcription factor resulted in the hyperactivation of Tconv and autoimmunity in mice (56).

It is well-accepted that glycolysis promotes Treg cell growth and migration at the cost of immune suppressive-function. Upon TCR ligation and distinct co-stimulations, transcription factor c-Myc and the hypoxia factor HIF-1α initiate the upregulation of genes encoding molecules important in the glycolysis pathway, whereas Bcl-6 directly downregulates glycolysis and associated pathways (57). Under hypoxic situations, HIF-1α prevent glucose-derived pyruvate from the mitochondrial oxidation, resulting in a gglycolytic shift, which leads to the inhibition of Treg function (43). Human Treg that lose FOXP3 expression upon *in vitro* stimulation preferably differentiated into Th2-like Treg (58), and the direct role of FOXP3 in suppressing type 2 cytokine production in Treg during Treg dysfunction has been confirmed by using the IPEX mutation M370I, a naturally occurring FOXP3 mutant derived from IPEX patients (59). The similar phenomenon was also observed in mice. Bcl6−/− Treg could produce Tconv-like levels of Th2 cytokines and are incapable of controlling Th2-type inflammation (60). The metabolic status of Th2-like Treg is still unclear. PTEN-mediated suppression of PI3K activity is critical for maintaining Treg suppressive function (44). PTEN-deficient Treg have increased glycolytic-rates and a significant reduction of FOXP3 expression. In Treg, restriction of mTORC1 signaling and glycolysis by Ndfip1, a coactivator of Nedd4-family E3 ubiquitin ligases, supports their suppressive function (45). Restriction of mTOR-pathway signaling also increases expression of transcription factor Bcl6 in Treg, which supports Treg stability and suppresses glycolysis potentiated by c-Myc and HIF-1α (57). Stimulation of the CTLA-4 and PD-1 pathways increases FOXP3 expression, stimulates OXPHOS and suppresses the glycolytic-flux in Treg (46). Curiously, glycolysis also supports

FOXP3 expression under certain conditions. The glycolytic enzyme Enolase-1 has been shown to be multifunctional. Under glycolytic circumstances, Enolase-1 is forced to the cytoplasm instead of binding FOXP3 in the nucleus, preventing it from suppressing FOXP3 expression in human Treg (47). Glycolysis also favors the activity of transcription repressor enhancer of zeste homolog 2 (EZH2), which promotes FOXP3 expression and is critical for the maintenance of Treg (48). We have shown that treatment of human Treg with a TNF receptor 2 agonist enhanced EZH2 expression, as well as lineage stability (49).

TGF-β functions in a complementary role to FOXP3 in promoting mitochondrial oxidative metabolism and inhibiting glycolysis. FOXP3 expression and OXPHOS activity are closely linked to human Treg intracellular ROS levels. ROS promotes FOXP3 stability in human Treg by increasing the activity of the transcription factor nuclear factor of activated T-cells (NFAT), which binds the CNS2 enhancer of FOXP3 (13, 50, 51). FOXP3 controls downregulation of glycolysis and transcription promoting factor Myc, induction of OXPHOS and increases the electron transfer NAD^+/NADH ratio. FOXP3 regulates T cell metabolism by suppressing Myc and glycolysis, enhancing OXPHOS and increasing nicotinamide adenine dinucleotide oxidation (52). Additionally, the AMPK-pathway inhibits mTOR signaling and thereby promotes OXPHOS (22). These adaptations allow Treg a metabolic advantage in low-glucose, lactate-rich environments, like in inflammation, as they resist lactate-mediated suppression of T cell function and proliferation. OXPHOS regulators are required for optimal Treg function. Deletion of OXPHOS key-regulators Pgc1α or Sirt3 abrogates Treg-dependent suppressive function. Myocyte enhancer factor 2 (Mef2) activity induces the expression of OXPHOS genes. Interestingly, inhibition of Mef2 with histone deacetylase 9 disproportionally affects Treg compared to Tcon, and deletion increases Treg suppressive function (13). As previously described, HIF-1α binds FOXP3 and thereby reduces suppressive function. Additionally, HIF-1α acts as a switch to promote Treg migration via promotion of glycolysis, at the cost of OXPHOS-driven immunosuppression (43). HIF-1α directs glucose to glycolysis and away from the mitochondria by blocking PDH, leaving mitochondrial metabolism dependent on fatty acids. Especially in hypoxic conditions, this diminishes suppressive function. Thus, OXPHOS regulators are required for optimal Treg function (13).

Treg also express high levels of AMPK, which promotes fatty acid oxidation and inhibits the mTOR mediated glycolysis (22). Stimulation of Treg effector molecules CTLA-4 and PD-1 and expression of FOXP3 suppresses glycolysis and promotes fatty acid oxidation *in vitro* (11). PD-1 actively promotes fatty acid oxidation by upregulation of fatty acid transporter CPT1. CTLA-4 and PD-1 activate PTEN to antagonize PI3K-signaling (6). DEP domain-containing mTOR-interacting protein (DEPTOR), a negative regulator of mTOR, has similar effects as partial inhibition of mTORC1 activity, shifting Treg metabolism toward OXPHOS while stabilizing FOXP3 expression and thereby securing Treg survival and suppressive function (53, 61).

Similar to fatty acid oxidation, fatty acid synthesis activity is required for Treg suppressive function although Treg are not dependent on fatty acid synthesis (62). The mevalonate pathway (**Figure 2**) aids the upregulation of the suppressive molecules CTLA-4 and ICOS (Inducible co-stimulator). Inhibition of the mevalonate pathway by disruption of mTOR by statins, genetic depletion of RAPTOR or inhibition of 25-hydroxycholesterol or 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) potently blocks human Treg suppressive activity, which can be reversed by addition of mevalonate (63, 64). Proper Treg cell function is also regulated by the ectoenzyme CD39 expression on human Treg. CD39 produces AMP (adenosine monophosphate) from ATP or ADP (adenosine diphosphate), which is further converted to extracellular adenosine by ectoenzyme CD73. Adenosine subsequently binds to adenosine 2A receptor (A2A) and facilitate Treg generation and suppressive function through adenosine-mediated immune suppression (65).

FOXP3 expression can also be regulated by post-translational modifications, which is closely linked to alterations in metabolism. Acetylation of FOXP3 prevents its degradation and is dependent on the availability of acetyl-CoA, a product of OXPHOS. However, fatty acid oxidation also antagonizes the stability of human Treg by promoting an increased NAD^+/NADH ratio, which increases the activity of deacetylase SIRT1 (silent mating type information regulation 2 homolog) (66). Short-chain fatty acids, like butyrate, stabilize Treg by preventing histone deacetylase from suppressing FOXP3 expression (28). IDO-mediated tryptophan metabolisms have inhibitory effects on Th1 and Th17 cells, while administration of the downstream tryptophan metabolite 3-hydroxy anthranilic acid (3-HAA) enhanced the percentage of Treg. IDO-deficient mice have reduced Treg levels (33), while IDO expression in plasmacytoid dendritic cells can induce tryptophan degradation and thereby support Treg generation and suppressive function.

In summary, glycolysis is associated with decreased Treg suppressive function. Lipid metabolism favorites Treg lineage stability. Several metabolites including purine, tryptophan, retinoic acid, and glutamine are crucial to support the induction of FOXP3 gene as well as its sustained stable expression (**Figure 3C**). The high expression of suppressive molecules on Treg like PD-1, CTLA-4, CD39, and AHR are crucial for Treg to sense the nutrient and energy change of its local milieu.

Treg METABOLISM IN PATHOLOGICAL CONDITIONS

Alterations in Treg numbers and function have been widely demonstrated in human autoimmune, infectious and allergic diseases and cancers (67). Decreased Treg numbers have been reported in patients with diabetes mellitus type II and has been contributed to both high glucose and high-density lipoprotein concentrations in blood (68). Circulating and visceral adipose Treg are reduced in obese individuals, inversely correlating with measures of adiposity, inflammation and glucose tolerance, enabling identification of subjects at increased metabolic and cardiovascular risk (69). PPARγ

signaling for energy homeostasis in Treg maintains adipose-tissue inflammatory tone and insulin sensitivity in lean adipose tissue, while disfunction attenuates insulin-sensitization (26). Interestingly, frequency and suppressive capacity of Treg remains unaltered following silencing of INSR, although attenuation of acute graft-vs.-host disease and multiple sclerosis has been observed in animal models (35). A role for Treg metabolism has also been described in cancer progression, with increases in circulating and tumor-infiltrating Treg strongly associated with advanced cancer stage and poor prognosis. The hypoxic tumor microenvironment supports Treg function and accumulation. Additionally, IDO-expression in the tumor microenvironment has been reported to support the conversion of conventional CD4+ T cells into Treg (70). IDO-expression associated Treg development has also been described in autoimmunity. IDO-deficient mice develop exacerbated experimental autoimmune encephalomyelitis (33) and IDO, together with ARG1, is lower expressed in blood cells of multiple sclerosis patients compared with healthy subjects (71). Increased IDO-expression is generally accompanied by increased mTOR expression, resulting in decreased Treg numbers and increased disease activity. mTOR-controlled pathways are likely to shape autoimmune responses in rheumatic diseases as well (72). In patients with systemic lupus erythematosus, mTORC1 is activated, and mTORC2 is inhibited, with activation of mTORC1 preceding disease flares, and being reduced during successful therapeutic intervention (72). Additionally, an important candidate gene for systemic lupus erythematosus susceptibility has been identified as a major regulator of mitochondrial metabolism and has been shown to reduce Foxp3 expression in Treg (73). Further research to Treg metabolism in pathological conditions, especially using metabolomic-approaches comparing various patient groups and healthy subjects, is imperative to aid a better understanding of the link between functional alterations of Treg and their intracellular metabolism.

EXPLOITING Treg METABOLISM FOR TOLERANCE-INDUCING THERAPIES

A better understanding of Treg metabolism and its distinction from other T cell subsets metabolism allows for the specific modulation of Treg *in vivo* or the improvement of adoptive Treg transfusion therapies. The encompassing goal of such therapies has been the induction of functional immunotolerance by harboring the natural specific immunosuppressive mechanisms without requiring damaging immunosuppressive drugs (74). Treg therapies could improve the current standard of care in the reduction of cost, increased availability, specificity to destructive immune responses, and applicability to different organs. However, before employing such therapies, it is imperative to understand the molecular mechanisms underlying critical Treg functionalities and to identify any factors that confound outcomes. For successful Treg therapy, it is rudimental to acquire a sufficient number of Treg, that these Treg migrate to their desired location and to subsequently have stable immunosuppressive functionality of Treg (75). Treg metabolism could be employed for this (**Figure 3**). Drugs for modulating cellular metabolism are already available, providing the field of immunometabolism with great opportunity to translate their findings to the clinic (**Table 2**).

Glycolysis is important for Treg migration. Although most research points toward a negative role for glycolysis in Treg proliferation, it has become clear that complete depletion of glucose from cell culture medium is detrimental for *in vitro* Treg proliferation and suppressive function. Inhibition of glycolysis reduces intracellular pyruvate levels, thereby preventing the conversion to acetyl-CoA via PDHK for mitochondrial oxidative metabolism (**Figure 2**) and can consequently reduce Treg proliferation (77). Interestingly, glycolysis can potentially be modulated in a Treg-specific manner, as Treg convert glucose to glucose-6-phosphate with a distinct isoform of hexokinase, hexokinase 1 (HK1) (19). At present, pharmacological interference with glycolysis can be obtained through 2-deoxy-D-glucose (2-DG), a glucose analog which is currently used at high concentrations in cancer therapy. 2-DG reportedly inhibits both Treg migration and proliferation (14, 15, 19, 35). Contrastingly, D-mannose, a C-2 epimer of glucose which also inhibits glycolysis, has been described to increase human Treg proliferation *in vitro* by the promotion of TGF-β activity which in turn leads to the increase of fatty acid oxidation (76). Mycophenolic acid, the active ingredient of the immunosuppressant mycophenolate mofetil currently in use for suppressing solid organ rejection, inhibits monophosphate dehydrogenase, an enzyme involved in the biosynthesis of guanine nucleotides, which follows the glycolysis-parallel pathway PPP (79). Interestingly, mycophenolic acid-enhanced expression of PD-1, CTLA-4, and FOXP3 and reduced Akt-mTOR and STAT5 signaling in human $CD4^+$ T cells.

Fatty acid oxidation is increased in proliferative Treg and inhibition thereof results in decreased Treg numbers (81). Moreover, oxidative metabolism is associated with and can be induced by Treg suppressive molecules such as TGF-β, CTLA-4, PD-1, and FOXP3. Dimethyl fumarate, a derivate of the TCA cycle intermediate fumarate, stimulates proliferation and development of Treg by supporting mitochondrial oxidative metabolism and FOXP3. Of note, dimethyl fumarate causes lymphopenia and selectively depletes highly glycolytic Teff while sparing oxidative naïve T cells and Treg (80). OXPHOS can be increased by the immunomodulatory metabolite rapamycin, which is used *in vitro* to expand Treg, potently suppresses T cell proliferation and increases Treg suppressive function *in vitro* and *in vivo*. This suggests that it is a valuable drug for adjuvant therapy to improve the efficacy of T(reg)-based immunosuppressive protocols (84). However, rapamycin also redirects Teff peripheral tissue trafficking and stimulates homing to lymph nodes by inhibition of mTORC1 (85). Whether rapamycin also redirects Treg migration is not yet established.

In a situation where blocking Treg function is preferred such as in the tumor milieu, the widely used anti-diabetic drug metformin can be employed. Metformin inhibits the electron transport chain and decreases mitochondrial ROS production, both AMPK-dependent and independent. Metformin increases Treg differentiation, most likely via

TABLE 2 | Metabolic modulators and their relevance for Treg proliferation, migration, and suppressive function.

Modulator	Pathway	Effect
2-DG	Inhibition of glycolysis	Decreases Treg numbers (14, 15, 19, 35) Decreases Treg migration (14, 19, 35)
D-mannose	Inhibition of glycolysis	Increases Treg numbers (76)
DCA	Inhibition of glycolysis	Increases Treg numbers (77)
DASA-58* and TEPP-46*	Inhibition of HIF-1α	Increases Treg numbers (78) Increases Treg suppressive function (78)
Mycophenolic acid	Inhibition of guanine nucleotide synthesis	Increases PD-1, CTLA-4, and FOXP3 expression of CD4+ T cells (79)
Dimethyl fumarate	TCA cycle	Increases Treg numbers (80)
UK5099*	Inhibition of TCA cycle	Decreases Treg numbers (19)
Rotenone	Inhibition of OXPHOS	Decreases Treg numbers (19) Decreases Treg suppressive function (13)
Oligomycin*	Inhibition of OXPHOS	Decreases Treg numbers (19) Decreases Treg suppressive function (22)
Metformin	Increase of FAO, inhibition of OXPHOS	Increases Treg numbers (23) Decreases Treg suppressive function (22)
AICAR	Increase of FAO	Increases Treg numbers (81)
Celastrol	Increase of FAO	Increases Treg numbers (82)
Etomoxir	Inhibition of FAO	Decreases Treg numbers (23)
C75*	Inhibition of FAS	Decreases Treg suppressive function (63)
Cerulenin*	Inhibition of FAS	Decreases Treg suppressive function (63)
Simvastatin	Inhibition of cholesterol synthesis	Decreases Treg numbers (63)
Amitriptyline	ASM	Increases Treg numbers (83)
BPTES*	Inhibition of glutaminolysis	Increases Treg numbers (31)
Rapamycin	Various	Increases Treg numbers (84) Redirects Teff migration (85) Increases Treg suppressive function (84)

2-DG, 2-deoxy-D-glucose; DCA, dichloroacetate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; BPTES, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide 3; HIF-1α, hypoxia-inducible factor 1-alpha; TCA, tricarboxylic acid cycle; OXPHOS, oxidative phosphorylation; FAO, fatty acid oxidation; FAS, fatty acid synthesis; ASM, acid sphingomyelinase.
**No experimental data specific for modulator available.*

suppressed activation of mTOR and HIF-1α and stimulation of AMPK and FOXP3 expression (23). Conversely, CPT1 inhibitor etomoxir inhibits fatty acid oxidation, which specifically reduced Treg differentiation and proliferation, although this might be caused by the off-target effects of etomoxir on metabolism. The chines herbal compound celastrol also has immunosuppressive capacities by promoting fatty acid oxidation via upregulation of CPT1 and AMPK expression. Additionally, celastrol has been indicated to facilitate FOXP3 expression and Treg cell generation (82). Pharmacological inhibition of acid sphingomyelinase (ASM), with a clinically used tricyclic antidepressant like amitriptyline, induces higher frequencies of Treg among T cells. This is due to ASM inhibition increasing cell death of T cells in general, while $CD25^{high}$ Treg are protected via IL-2 (83). Further, ASM deficient pTreg have less Akt activity and RICTOR levels compared with control pTreg. Inhibitors of the rate-limiting enzyme HMGR impairs Treg proliferation and function whereas addition of mevalonate, the metabolite downstream of HMGR restores Treg-mediated suppression (63), suggesting that manipulation of lipid biosynthesis, in particular via the mevalonate pathway, would result in Treg functional disruption.

Various studies have described the alterations of metabolic pathways and key metabolic byproducts in several autoimmune disorders. Disease-specific metabolic changes in overall glycolytic activity and oxidative state have been reported in rheumatoid arthritis and multiple sclerosis. In multiple sclerosis, impaired proliferation is suggested to be a consequence of increased levels of circulating leptin. The glutaminolysis pathway has been suggested as a biomarker for disease severity (86). In solid organ transplantation, it is reported that the metabolic environment might influence immune responses and overall transplantation outcome. Lee et al. have shown that by simultaneously blocking glycolysis and the glutamine pathway in the inflammatory transplantation microenvironment, allo-specific Teff responses could safely be reduced while preserving immunoregulation (87). Indeed, both glycolysis and glutamine are associated with a pro-inflammatory phenotype and non-essential for Treg suppressive function, although not irrelevant for Treg proliferation. Wawman and colleagues have described the importance of the hepatic microenvironment in transplantation. The continuous exposure of metabolites and nutrients influences lineage fitness, function, proliferation, migration, and survival of Treg (88). This paves the way for safe and specific novel approaches to modulate the inflammatory environment, for example with tissue-specific accumulation of nanobiologicals (89).

CONCLUDING REMARKS

Knowledge on the role of metabolism in immune cells is rapidly expanding and has increasingly been acknowledged as a potential target for therapies aimed at modulating the immune system either to enhance or suppress immunological responses. Metabolic profiles in Treg are distinctly different between proliferation, migration, and suppressive function, and can be modulated using readily available metabolic and immunomodulatory drugs. However, as metabolic modulation impacts Treg differently throughout their functional profiles, we lack precise insight into the regulatory switches. While modulation of some aspects of glycolysis keeps Treg proliferation and suppressive function intact, an overall increase in glycolysis inhibits Treg suppressive capacity, however, this supports Treg migration. Contrary, oxidative metabolism is crucial to support Treg suppressive function but appears less relevant for migratory behavior. Taken together, this demonstrates the counter-regulation of Treg cell metabolism by pro- and anti-inflammatory signals.

Current technologies for investigating immunometabolism of Treg are limited. Glycolysis and OXPHOS can be measured using Seahorse technology or fluorescent uptake of metabolites, but for the application of these techniques, a high number of cells are required. In the case of Treg, comprising only 1–5% of circulating CD4+ cells, it means that *ex vivo* expansion is almost unavoidable, which may change their metabolic profile. Studying the metabolism of tissue resident-Treg is challenging for similar reasons, with the added challenge purification from tissues brings. To enable research on the metabolism of migrating Treg, improved *in vivo* Treg tracking techniques are required, just as advanced *in vitro* techniques that can combine the technology of migration chambers and metabolism assessment. Also, it should be appreciated that the difference in metabolic profiles, in general, is context-dependent. Distinct Treg proliferative behavior has been reported between *in vitro* and *in vivo* experiments, which indicates the milieu of the microenvironment to be of crucial importance for linking metabolism to immune cell function.

Overall, although exploitation of Treg metabolism seems promising and results could be translated into practice relatively easily, significant challenges are still to be faced. Future research using novel -omics approaches will offer further insight into the molecular mechanisms underlying Treg metabolism. These insights will guide new research on the improvement of current Treg therapies. Given the reliance of specific T cell subsets on certain metabolic pathways, it is possible that the ability of subsets to use and regulate specific pathways is differently regulated and differently metabolic sensitive. Although a simplified reductionistic approach to immunometabolism makes the concept more comprehensible, it is imperative to keep in mind that metabolism is complex, and pathways intertwine and impact each other at many different levels. From a pragmatic point of view, immunometabolism presents excellent possibilities for modulating immune responses, as drugs altering the metabolic state of cells are readily available.

Collectively, the data described in this literature review emphasizes the link between immune cell metabolism and Treg profiles, and underline the importance of understanding the machinery providing the energy required for immune cell functions and could have implications in natural mechanisms to increase Treg suppressive function, like in the transplant setting and autoimmunity. The fields of immunometabolism and Treg research are both burgeoning and combining them might prove useful for patients' benefit in the near future.

AUTHOR CONTRIBUTIONS

RK, IJ, HK, and XH contributed conception, design of the study, and wrote sections of the manuscript. RK organized the database and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02839/full#supplementary-material

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REVIEW
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Metabolic Control of Treg Cell Stability, Plasticity, and Tissue-Specific Heterogeneity

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Regulatory T (Treg) cells are crucial for peripheral immune tolerance and prevention of autoimmunity and tissue damage. Treg cells are inherently defined by the expression of the transcription factor Foxp3, which enforces lineage development and immune suppressive function of these cells. Under various conditions as observed in autoimmunity, cancer and non-lymphoid tissues, a proportion of Treg cells respond to specific environmental signals and display altered stability, plasticity and tissue-specific heterogeneity, which further shape their context-dependent suppressive functions. Recent studies have revealed that metabolic programs play pivotal roles in controlling these processes in Treg cells, thereby considerably expanding our understanding of Treg cell biology. Here we summarize these recent advances that highlight how cell-extrinsic factors, such as nutrients, vitamins and metabolites, and cell-intrinsic metabolic programs, orchestrate Treg cell stability, plasticity, and tissue-specific heterogeneity. Understanding metabolic regulation of Treg cells should provide new insight into immune homeostasis and disease, with important therapeutic implications for autoimmunity, cancer, and other immune-mediated disorders.

Keywords: metabolism, Treg cell, Foxp3, stability, plasticity, tissue-specific heterogeneity

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INTRODUCTION

Regulatory T (Treg) cells are critical for the establishment of peripheral tolerance, with altered Treg cell function leading to autoimmune disease and immunopathology (1, 2). Treg cells constitutively express CD25, the α subunit of IL-2 receptor, and require continuous IL-2 signals for homeostasis and function (1–5). The transcription factor forkhead box P3 (Foxp3) is essential for specifying the lineage and suppressive function of Treg cells (1, 2). The majority of peripheral Treg cells originate from the thymus and are known as thymus-derived Treg (tTreg) cells (6–8). Treg cells may also differentiate from naïve $CD4^+$ T cells in the periphery [called peripherally-derived Treg (pTreg) cells] or *in vitro* after stimulation in the presence of TGF-β and IL-2 (termed iTreg cells) (6, 9, 10), which are distinguished from tTreg cells by the lack of Helios and neuropilin-1 expression (11–13). In addition, epigenetic modifications of the *Foxp3* locus differ between tTreg and pTreg cells (6, 10). How these Treg cells arise and contribute to Treg cell suppressive function in different contexts has remained an important question for the field.

Recent advances have highlighted the important role of metabolism in immune cells, including Treg cells (14, 15). Initial studies showed that iTreg cells and conventional effector T helper cells (Th1, Th2, and Th17) require fatty-acid oxidation (FAO) and glycolysis, respectively, for their proliferation, differentiation, and survival (16). More recent analysis has shown that

Foxp3 expression likely contributes to these effects (17–19). However, Treg cells *in vivo* are more metabolically active than conventional naïve T cells and undergo increased levels of proliferation balanced by apoptosis (20–22). Also, dietary nutrients and metabolites serve as important environmental factors that influence Treg cell function (23). Intracellular metabolites and metabolic pathways also modulate the expression of Foxp3, as well as Treg cell transcriptional programs and functional plasticity (20, 21, 23). In particular, nutrient-fueled mTORC1 activation promotes metabolic reprogramming in Treg cells *in vivo*, with increased lipogenesis and mevalonate pathway-dependent cholesterol biosynthesis to support Treg cell proliferation and function (22, 24). However, inappropriate mTORC1 activation and unconstrained glycolysis in Treg cells lead to decreased Foxp3 expression and reduced Treg cell suppressive activity, indicating that cellular metabolism plays essential roles for regulating Foxp3 stability and Treg cell function (18, 25–27). In this review, we summarize the recent advances that have defined how environmental metabolites and nutrients, as well as cell-intrinsic metabolic programs, orchestrate Treg cell function by affecting stability, plasticity, and tissue-specific heterogeneity.

METABOLIC REGULATION OF TREG CELL LINEAGE STABILITY

Dysfunctional mutations in the *Foxp3* gene result in fatal autoimmunity with Scurfy phenotype in mice and IPEX (Immuno-dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome in humans due to altered Treg cell development (28, 29). However, maintaining Foxp3 expression is also essential for Treg cell function. The majority of Treg cells are a stable population under steady state or upon transfer into environments that contain T cells (30, 31). More recently, the concept of Treg cell stability, which is defined as the ability to maintain Foxp3 expression and resist acquiring pro-inflammatory effector functions during inflammation, has emerged as a crucial determinant of Treg cell function in selective contexts (32–34). For example, Treg cells display considerable loss of stability *in vitro* when stimulated with proinflammatory cytokines, including IL-6 and IL-4 (35, 36). The resultant $Foxp3^{-}$ cells are referred to as "exTreg" cells (35), which are also observed in autoimmune mouse models (37). Adoptive transfer of purified $Foxp3^{+}$ Treg cells into lymphopenic recipients that lack conventional T cells also results in a dramatic loss of Foxp3 expression (30, 37, 38). These $Foxp3^{-}$ cells acquire the expression of inflammatory cytokines and fail to mediate immune suppression (30, 37, 38). Interestingly, the unstable Treg cells are mostly limited to $CD25^{lo}Foxp3^{+}$ subset, raising the possibility that a small portion of Treg cells are inherently prone to becoming unstable *in vivo* (30). Further research using fate-mapping mouse models has shown that some exTreg cells are from activated T cells that have transiently expressed Foxp3 and failed to fully differentiate into Treg cells (39), thus establishing stability as a context-dependent regulator of inflammation and peripheral tolerance.

The molecular mechanisms that prevent the loss of Foxp3 expression have been extensively studied, with the current understanding that Foxp3 expression is maintained through transcriptional, epigenetic and post-translational regulation. First, multiple transcription factors regulate *Foxp3* gene expression by directly binding to *Foxp3* gene promoter, such as STAT5, NFAT, and Foxo1. In addition, the *Foxp3* gene locus contains conserved non-coding sequence (CNS) elements, which recruit transcription factors to regulate gene expression (40–42). For example, CNS1 responds to TGF-β and recruits Smad3 (43); CNS2 recruits STAT5 (35), NFAT (44), RUNX (45), and CREB (46), among others; and the NF-κB signaling component c-Rel binds to CNS3 (47). Second, CNS2 contains a Treg cell-specific demethylated region (TSDR) (48), which is largely demethylated in tTreg cells and partially methylated in iTreg or pTreg cells (41, 42, 49, 50). The demethylated TSDR allows for recruitment of transcription factors, such as Foxp3 itself, CREB, and Ets-1, to stabilize Foxp3 expression (46, 51, 52). Third, acetylation, phosphorylation and ubiquitination have been identified to orchestrate Foxp3 protein stability (42). In particular, recent studies have established a critical role of metabolism in regulating Treg cell stability through interplaying with the established mechanisms of transcriptional, epigenetic, and post-translational control of Foxp3 expression (**Figure 1**). Below, we summarize the progress in metabolic regulation of Treg cell stability. We first discuss how environmental nutrients and metabolites influence Foxp3 stability. Then, how intrinsic cellular metabolism modulates Treg cell lineage identity is detailed. Finally, the signaling mechanisms for establishing metabolism-dependent control of Foxp3 expression are described.

Environmental Nutrients and Metabolites

Multiple dietary nutrients, vitamins, and metabolites can directly modulate Foxp3 expression in Treg cells. Among them, the vitamin A metabolite all-*trans* retinoic acid (RA), produced by specific dendritic cell (DC) subsets, directly and indirectly modulates *Foxp3* expression (53–56). RA directly increases the activation of extracellular-related kinase (ERK) signaling to promote *Foxp3* expression (56). RA also increases histone methylation and acetylation of the promoter and CNS at the *Foxp3* gene locus (56). RA indirectly promotes TGF-β-mediated $Foxp3^{+}$ Treg cell conversion by relieving inhibition from $CD44^{hi}$ memory T cells (57). Specifically, $CD44^{hi}$ memory T cells release a series of inflammatory cytokines, such as IL-4, IL-21, and IFNγ, which act synergistically to inhibit TGF-β-induced Foxp3 expression; however, RA suppresses these pro-inflammatory cytokine programs and therefore stabilizes Foxp3 expression (57). RA can also prevent the loss of *FOXP3* expression during human Treg cell expansion and in inflammation, with superior efficacy as compared with rapamycin (an mTORC1 inhibitor) that is known to promote stable Foxp3 expression (55).

In addition to vitamin A, vitamins C and D have been directly linked to the regulation of Foxp3 expression. Recent research has established roles for vitamin C in immune cell function, including its ability to stabilize *Foxp3* expression by demethylation of CNS2 region in iTreg cells (58, 59). Specifically, the CpG motifs of

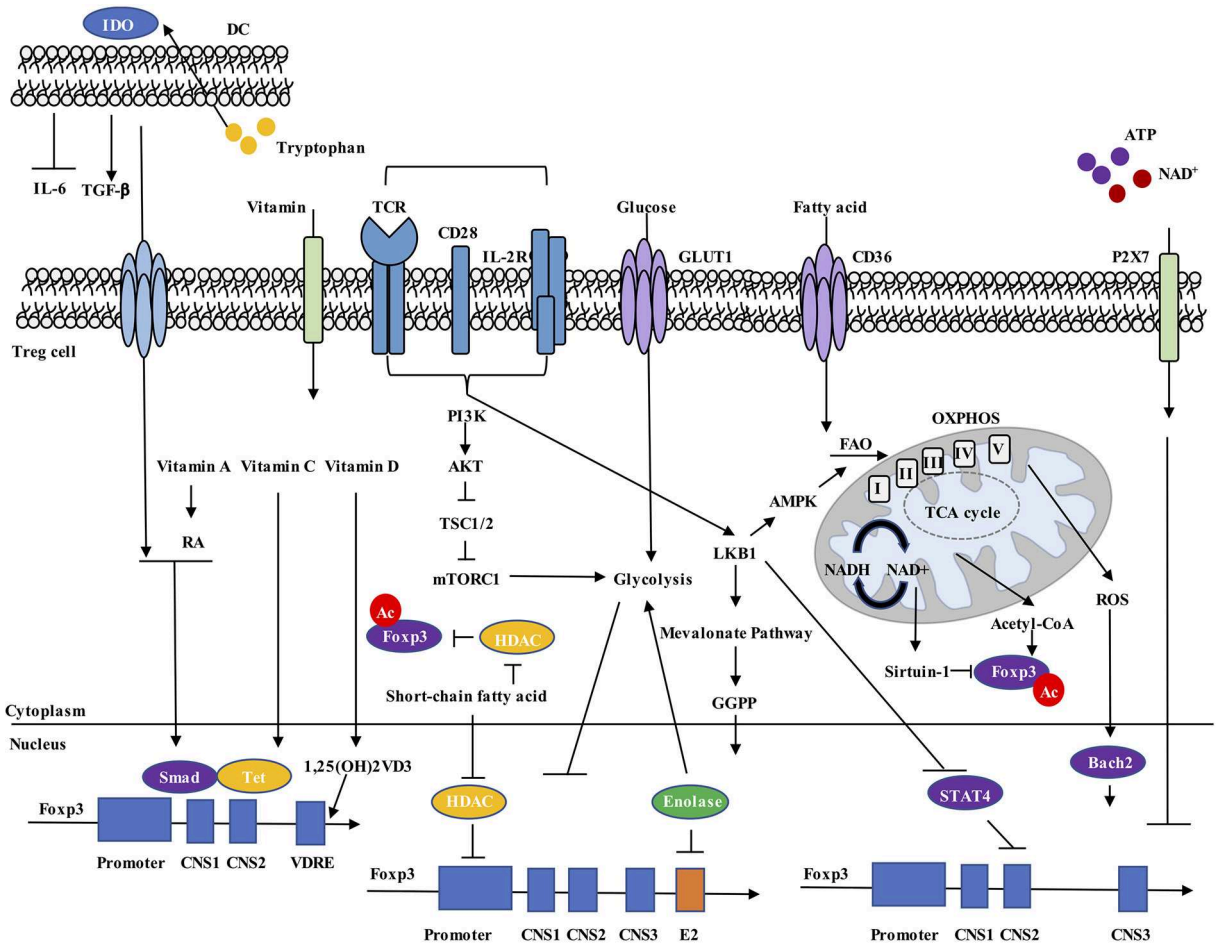

FIGURE 1 | Metabolic regulation of Foxp3 expression. Environmental metabolites, intracellular metabolic intermediates, and signaling pathways all regulate Foxp3 expression in Treg cells. (1) Dendritic cells (DCs) express IDO, drive tryptophan metabolism to promote TGF-β and inhibit IL-6 production, and increase Foxp3$^+$ Treg cell generation. (2) Vitamin A metabolite RA, together with TGF-β-induced Smad activation, increase *Foxp3* expression. Vitamin C stabilizes Foxp3 expression through maintaining demethylated state of *Foxp3* CNS2 region by Tet methylcytosine dioxygenase. Vitamin D3 metabolite 1,25$(OH)_2VD_3$ increases *Foxp3* gene expression by binding to VDRE region. (3) Extracellular ATP and NAD$^+$ released by cell lysis activate the P2X7 receptor and induce Treg cell instability. (4) Increased cellular ratio of metabolites NAD$^+$/NADH activates the deacetylase activity of Sirtuin-1 and destabilizes Foxp3 protein, while acetyl-CoA increases acetylation level of Foxp3 protein and promotes its stabilization. (5) ROS promotes SENP3-driven Bach2 deSUMOylation and nuclear localization, thus stabilizing Foxp3 expression. (6) Short-chain fatty acids stabilize Foxp3 expression, possibly by inhibiting HDAC-mediated suppression of *Foxp3* gene expression and Foxp3 protein deacetylation. (7) Unconstrained activation of mTORC1 and glycolysis inhibit Foxp3 expression and reduce suppressive activity of Treg cells. (8) LKB1 prevents STAT4 activation and binding to CNS2 of *Foxp3* gene, thus preventing the destabilization effect by inflammatory cytokines. LKB1 also regulates Foxp3 expression through activation of mevalonate pathways. RA, all-trans retinoic acid; VDRE, vitamin D response element; CNS2, conserved non-coding sequence 2; NAD, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; HDAC, histone deacetylase.

CNS2 in iTreg cells are partially methylated (48, 60), and CNS2 becomes demethylated after treatment with vitamin C, whose effect is mediated by the Tet family demethylase proteins (58, 59). Deletion of Tet2 blocks the demethylation effect of vitamin C (58, 59). Deletion of Tet2/Tet3 in Treg cells indeed leads to unstable Foxp3 expression (61, 62). Vitamin D3 is synthesized in the skin in response to ultraviolet light or acquired from diet, and the vitamin D3 metabolites, 25-dihydroxyvitamin D3 [25(OH)VD_3] and the active form 1,25$(OH)_2VD_3$, can promote *Foxp3* expression in TCR and IL-2-activated CD4$^+$ T cells (63, 64). Subsequent analysis has revealed the presence of vitamin D response element (VDRE) in the intronic conserved CNS region (+1714 to +2554) of the human *FOXP3* gene as a functional enhancer, which underlies how vitamin D3 induces *FOXP3* expression (65). However, the specific transcriptional regulation mechanisms of VDRE on *FOXP3* gene remain to be ascertained.

Other metabolites also regulate Foxp3 expression in Treg cells, such as those from tryptophan and purine metabolism. DCs that express the enzyme indoleamine 2,3-dioxygenase (IDO) can catabolize tryptophan. The IDO-dependent catabolic program subsequently induces Foxp3$^+$ Treg cell generation through inhibition of IL-6 production by DCs (66, 67). Mice treated with the tryptophan metabolite 3-hydroxyanthranilic acid

(3-HAA) increase expression of TGF-β in DCs, display increased percentage of Treg cells, reduced frequencies of Th1 and Th17 cells, and ameliorated development of experimental autoimmune encephalomyelitis (EAE) (68). Tryptophan also serves as the precursor for *de novo* nicotinamide adenine dinucleotide (NAD^+) synthesis (69), which is also regenerated from NADH by reduction of pyruvate to lactate during activation of glycolysis. The NAD^+/NADH ratio directly regulates the activity of deacetylase Sirtuin-1 (70). Since acetylation improves Foxp3 protein stability (71), Sirtuin-1 post-translationally impairs the acetylation and stability of Foxp3 (72). Foxp3 expression is also regulated by metabolites from extracellular purine metabolism, which orchestrates the balance of proinflammatory ATP and anti-inflammatory adenosine. Extracellular ATP and NAD^+ that are released by cell lysis or non-lytic mechanisms during cell damage and inflammation can activate the P2X7 receptor and induce T cell death. Treg cells highly express P2X7 receptor, which upon activation can limit Foxp3 stability and enhance Treg cell conversion into Th17 cells (73). Intriguingly, Treg cells express high levels of the ectonucleotidases CD39 and CD73 on the surface, which convert excess extracellular ATP into immunosuppressive adenosine to relieve the harmful effect of extracellular ATP and increase their suppressive function (74). The results suggest that purine metabolism could be important for Treg cells to maintain stability. Overall, we still know little about how nutrients and metabolites regulate Treg cell stability, especially *in vivo*, which may be uncovered by using metabolomics techniques.

Cellular Metabolism

Cellular metabolism is also closely related to Treg cell stability. Compared to Th1, Th2, and Th17 cells, Treg cells are less reliant on glycolysis and use mitochondrial metabolism and oxidative phosphorylation (OXPHOS) for energy production (16). *In vitro* studies reveal that expression of Foxp3 reprograms T cell metabolism by suppressing glycolysis and enhancing OXPHOS (17, 18). The effector molecules CTLA4 and PD-1 on Treg cells also suppress glycolysis in T cells (75, 76). Several studies have indicated that elevated glycolysis may be detrimental to Treg cell induction, as inhibition of glycolysis promotes the induction of Foxp3 expression in response to TGF-β and IL-2 stimulation (77, 78). In addition, deletion of HIF-1α, a transcription factor that can promote glycolysis, also leads to increased Foxp3 induction (78). *In vivo*, transgenic mice expressing Glut1 (Glut1-tg) have a greater proportion of $CD25^{lo}Foxp3^+$ cells than those from wild-type mice (18). Further analysis has demonstrated that Glut1-tg expression reduces Foxp3 expression in iTreg cells and *in vivo* during intestinal inflammation. Analysis of key metabolism-related proteins has also illustrated that excessive glycolysis can lead to reduced Treg cell stability. For instance, c-Myc promotes glycolysis in T cells (79), whose activity was recently shown to be inhibited by autophagy to maintain Treg cell stability (25). Furthermore, specific deletion of phosphatase and tensin homolog (PTEN) in Treg cells also leads to PI3K/Akt-mediated hyperactivation of glycolysis, a greater proportion of $CD25^{lo}Foxp3^+$ cells similar as Glut1-tg mice, and increased methylation of the TSDR region of *Foxp3* gene (26, 27). These studies together demonstrate that unrestrained glycolysis results in reduced Treg cell stability. Of note, a recent study in human Treg cells has demonstrated that the glycolytic enzyme Enolase-1 binds to the *FOXP3* promoter and its CNS2 region, and represses the transcription of a splice isoform containing Exon-2 (FOXP3-E2), which is important for Treg cell suppressive activity (80). Glycolysis drives Enolase-1 to translocate out of nucleus and relieves the repression of transcription of FOXP3-E2 (80). Moreover, recent studies have established that glycolysis also increases Treg cell migration (81). It is reasonable to speculate that Treg cells precisely calculate and balance cellular glucose consumption, where heightened glycolysis increases Treg cell proliferation or migration to fill the niche and expand the pool *in vivo*, but this activity is balanced by other metabolic programs (for example, OXPHOS) to maintain lineage stability and suppressive activity. Intriguingly, TLR signals in Treg cells have been shown to promote PI3K/Akt signaling, and increase glycolysis and proliferation, while reducing suppressive function (18). Thus, we propose that Treg cell stability and function are under precise control of glycolysis. However, the detailed mechanisms whereby glycolysis interplays with Foxp3 expression remain to be ascertained.

As noted above, several recent studies have demonstrated crucial roles for mitochondrial metabolism and OXPHOS for Treg cell suppressive activity both at steady state and in the tumor microenvironment (82–85). Treg cells display greater mitochondrial mass and higher levels of reactive oxygen species (ROS) compared with conventional T cells (82), likely produced from OXPHOS. ROS is reported to increase the SUMO-specific protease 3 (SENP3) stabilization, and trigger Bach2 deSUMOylation (86). DeSUMOylation of BACH2 prevents its nuclear export, thus maintaining Foxp3 expression and Treg cell stability (86). Moreover, Treg cell-specific deletion of mitochondrial transcription factor A (Tfam) (promotes synthesis of mitochondrial DNA-derived proteins), can destabilize Foxp3 expression, which is associated with enhanced methylation in the TSDR of *Foxp3* locus in Treg cells under inflammatory contexts (87), whereas Tfam is dispensable for Foxp3 stability in the absence of inflammation (85). These findings are in agreement with a recent study that showed specific deletion of mitochondrial complex III impairs suppressive function without altering Foxp3 expression in Treg cells (84). Thus, OXPHOS in Treg cells seems to enforce Treg cell function independently of regulating their stability.

A growing area of interest is the regulation of OXPHOS by extracellular nutrients in Treg cells. As upregulation of glycolysis can be detrimental to Treg cell function, several studies have instead focused on the roles of fatty acids and FAO for the regulation of Treg cell function and stability. FAO involves the degradation of fatty acids by the sequential removal of two-carbon units from the acyl chain to produce acetyl-CoA, which enters the mitochondrial tricarboxylic acid (TCA) cycle to regulate mitochondrial OXPHOS and other functions. Short-chain fatty acids can indeed stabilize Foxp3 expression, possibly by inhibiting the expression of histone deacetylases (HDACs), such as HDAC6 and HDAC9 that can

destabilize Foxp3 protein stability (88). However, the roles of HDACs is likely complex, as they may also orchestrate acetylation status of other transcription factors, like STAT5 that induces and sustains *Foxp3* gene expression (89). Other studies have also revealed that acetyl-CoA levels contribute to Foxp3 expression through the post-translational control of protein acetylation and protein stability in Treg cells (90). However, FAO may serve only context-dependent roles in Treg cells. Carnitine palmitoyl-transferase 1A (Cpt1a) is a protein found in the outer mitochondrial membrane that catalyzes the esterification of long-chain acyls with carnitine to form acyl-carnitine and is considered to be the rate-controlling for long-chain FAO. The frequency and total number of Treg cells in mice lacking *Cpt1a* in $CD4^+$ cells or in $Foxp3^+$ Treg cells are comparable in different tissues (91). *Ex vivo*-isolated Treg cells lacking *Cpt1a* display normal Foxp3 expression levels and similar mitochondrial oxidative capacity relative to their wild-type counterparts. As the study is unable to rule out the possible role of medium-chain and short-chain fatty acids, the effects of fatty acid metabolism on Foxp3 expression and stability require further investigation.

Metabolic Signaling

Several signaling pathways that impact T cell metabolism have been identified. Among them, PI3K/Akt and LKB1/AMPK (AMP-activated protein kinase) signaling pathways play central roles. PI3K catalyzes the conversion of PtdIns-4,5-P_2 (PIP2) toward PtdIns-3,4,5-P_3 (PIP3) and activates kinases with Pleckstrin homology (PH) domains, most notably Akt (92). PI3K/Akt signaling is activated by upstream TCR and IL-2 signaling in Treg cells (93). Akt also directly phosphorylates the Foxo (Foxo1 or Foxo3a) transcription factors and blocks their nuclear translocation (94). Stable tTreg cells display hypoactivation of Akt, resulting in enhanced nuclear Foxo abundance on the promoter regions of *Foxp3*, leading to stable Foxp3 expression (95–97). These observations are consistent with those showing that Foxo activity limits glycolysis in T cells via impairing c-Myc function (98). Ligation of neuropilin-1 by Sema4a promotes Treg cell stability through PTEN-dependent inhibition of Akt activity (99), which facilitates Foxo nuclear localization and thereby increases Foxp3 expression (99). These results indicate an indispensable role of the Akt/Foxo pathway in orchestrating Treg cell stability.

Akt can also indirectly affect the activation of the mTOR complexes, mTORC1 and mTORC2, which integrate upstream metabolic signals for metabolic programming (100). Akt phosphorylates TSC2 to relieve TSC complex inhibition on mTORC1, which is a critical driver of glycolysis through augmenting the expression of glucose transporters, such as Glut1, or transcription factors, including c-Myc (100, 101). mTOR inhibition by rapamycin drastically enhances TGF-β-induced Foxp3 expression *in vitro* (102), indicating that Treg cells require low mTORC1 activity for induction of Foxp3 expression. However, deletion of mTORC1 in $Foxp3^+$ Treg cells does not affect Foxp3 expression on a per cell basis *in vivo* (22), whereas conditional deletion of TSC1 in Treg cells leads to impaired Foxp3 expression and heightened IL-17 production under inflammatory conditions (103). Thus, unconstrained mTOR activation can have deleterious impacts on Treg cell stability. Several studies have also investigated the role of mTORC2 in Treg cell stability and function. Rictor (the obligate protein for mTORC2) deletion in Treg cells results in no obvious abnormalities (22). Rictor-deficient T cells also retain their capacity to become $Foxp3^+$ iTreg cells (104, 105). Thus, mTORC2 has a less dominant function *in vivo* and *in vitro* than that of mTORC1 at steady state. However, upon Foxp3 deficiency, mTORC2 is responsible for augmented aerobic glycolysis and OXPHOS in Treg cells, and deletion of Rictor could restore the suppressive activity of Foxp3-deficient Treg cells (106). TCR and IL-2 are two of the most important upstream drivers for mTORC1 activation in Treg cells. Co-stimulation with TCR and IL-2 *in vitro* can relieve the suppression of PTEN on Akt-mTOR activation in Treg cells, reverse the anergic state of freshly-isolated Treg cells and promote their proliferation (107). How Treg cells maintain mTOR activation-mediated expansion and prevent unconstrained mTOR activation-induced instability *in vivo* warrants further investigation. However, it should be noted that hormones, such as leptin, may allow for temporal tuning of mTOR activation to promote appropriate expansion of Treg cells without affecting Foxp3 stability (108). Thus, a key future direction will be to dissect the upstream metabolic inputs that promote mTOR activity in Treg cells, toward understanding the effect of environmental cues and the mechanisms by which they are transmitted to impact Treg cell function.

The AMPK pathway, which is activated in response to cellular stress (e.g., AMP/ATP ratio), suppresses mTOR signaling and promotes mitochondrial OXPHOS rather than glycolysis (109–111). The AMPK agonist AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) strongly enhances Treg cell expansion (112). Activation of AMPK signaling by metformin-induced FAO also promotes Treg cell generation *in vivo* (16). iTreg cells have high levels of activated AMPK and FAO (16), likely due to the ability of AMPK to modulate Cpt1a activity and increase fatty acid import into mitochondria for β-oxidation (110). The serine-threonine kinase LKB1 is activated in response to TCR signals and can directly phosphorylate and activate AMPK (111, 113). LKB1 promotes mitochondrial fitness and FAO in Treg cells (113); however, these events appear to be AMPK-independent (113–116), suggesting other downstream LKB1 targets as important regulators of metabolic programming in Treg cells. In addition, recent analysis of LKB1-deficient Treg cells demonstrates that LKB1 enhances Foxp3 expression by preventing CNS2 methylation (114), and by activation of the mevalonate pathway that generates many metabolites, including cholesterol and the isoprenoid geranylgeranylpyrophosphate (GGPP) (115). Specifically, LKB1 prevents STAT4 activation and binding to CNS2, thus maintaining Foxp3 stability in response to STAT4-inducing inflammatory cytokines (114). Moreover, mevalonate or GGPP treatment restores the function and stability of LKB1-deficient Treg cells (115). The precise mechanisms that control AMPK and LKB1 activation in Treg cells require further study.

SHAPING TREG CELL PLASTICITY BY METABOLISM

Unlike loss of stability, "plastic" Treg cells tend to retain Foxp3 expression, but acquire the expression of transcription factors associated with effector T cell programs (called Th-like Treg cells). Plasticity is essential for Treg cells to exert specific suppressive activity toward selective types of inflammation and various environmental conditions. Specifically, Treg cells express the transcription factor T-bet to suppress type-1 inflammation, and acquire IRF4 and STAT3 to inhibit type-2 and type-17 inflammation, respectively (117–120). Gata3 expression by Treg cells is also important for suppression of type-2 inflammation in barrier sites like the skin and intestine (121, 122). In other studies, loss of Gata3 in Treg cells is associated with reduced Foxp3 expression and increased expression of RORγt during inflammation (123, 124), while co-deletion of T-bet and Gata3 directly leads to severe autoimmune-like disease and impaired Treg cell function (125). Moreover, Bcl6 is induced to generate T-follicular regulatory (Tfr) cells, which control germinal center responses (126–128). It is important to note that subsequent studies have revealed that these Th-like Treg cells may display impaired suppressive function in certain contexts (129–132). Th-like Treg cells normally display demethylated state on Foxp3 TSDR region (129). However, in certain inflammatory contexts, the epigenetic status could be altered by the presence of environmental cytokines, which may allow these cells to express proinflammatory cytokines (132). Thus, it remains unclear why Th-like Treg cells are suppressive in some contexts but pro-inflammatory in others. Notably, there is considerable controversy about whether instability is a separate cellular state from plasticity (33, 34). Both instability and plasticity have been observed in various autoimmune diseases in mice and humans, but the mechanistic connection between instability and plasticity in disease settings remains incomplete. Whether Th-like Treg cells represent a transient stage toward becoming Foxp3$^-$ Treg cells requires further investigation.

The best characterized Th-like Treg cells are Th1-like Treg cells, which express T-bet and CXCR3 (117). Th1-like Treg cells have been observed in autoimmune (Sjögren syndrome) (133) and tumor models. In fact, increased expression of IFN-γ by Treg cells can markedly improve checkpoint blockade therapy (134, 135). The dominant effect of IFN-γ$^+$ Treg cells in these tumor models indicates that these cells not merely lose suppressive function but gain anti-tumor effector activity. In humans, IFN-γ$^+$ Treg cells are found in patients with relapsing/remitting multiple sclerosis (RRMS), type 1 diabetes and autoimmune hepatitis (129, 132, 136). *In vitro*, IL-12 stimulation induces T-bet, CXCR3, CCR5, and IFN-γ expression in Treg cells, which maintain a demethylated TSDR and *FOXP3* expression (129, 131). These results underscore a critical role of IL-12 signaling in promoting Th1-like Treg cell generation. The observations are consistent with those that T-bet expression is further induced in type-1 inflammation, but studies using mouse models have shown that T-bet and IFN-γ are not co-expressed in Th1-like Treg cells as Th1 cells, possibly due to delayed induction of IL-12 receptor component IL-12Rβ2 and IL-12 signaling (117). Thus, how IL-12 can differentially regulate Th1-like programming in Treg cells as compared with Th1 cells remains an interesting question to address.

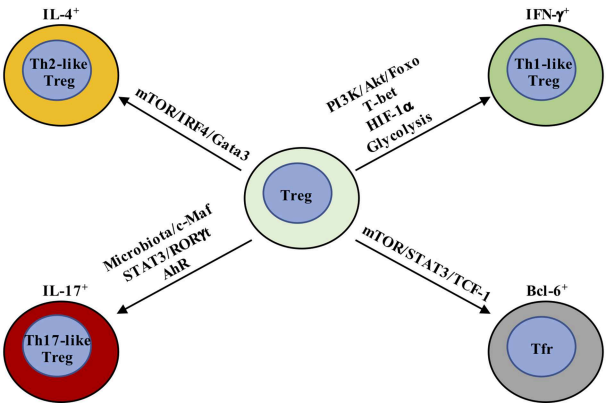

FIGURE 2 | Metabolic and transcriptional regulation orchestrates Treg cell plasticity. Th1-like Treg cells are T-bet$^+$ and express IFN-γ, whose differentiation is driven by IL-12-mediated PI3K/Akt/Foxo activation. In addition, HIF-1α increases IFN-γ expression by enhancing glycolysis and directly binding *Ifng* gene to promote Th1-like Treg cell differentiation. Th2-like Treg cells are characterized by the expression of Gata3 and IRF4 and production of IL-4 and IL-13. TCR and downstream mTOR activation promote IRF4 expression to facilitate Th2-reprogramming in Treg cells. Th17-like Treg cells express RORγt in addition to Foxp3. IL-6 and downstream STAT3 activation are critical for the generation of IL-17-producing Treg cells. The tissue in which Th17-like Treg cells have been best characterized is the intestine, where Treg cells adapt to the local environmental cues, like microbiota and AhR ligands. T-follicular regulatory (Tfr) cells co-express Bcl-6 and Foxp3. mTORC1 promotes the phosphorylation of STAT3 and induces the expression of TCF-1 to drive Bcl-6 expression.

Metabolic pathways are emerging regulators of Treg cell plasticity (**Figure 2**) (95, 113, 131, 137). For instance, PI3K/Akt/Foxo signaling plays an important role in regulating IL-12-induced IFN-γ production in Treg cells (131), but also promotes metabolic programming as discussed below. Freshly-isolated IFN-γ$^+$ Treg cells display increased expression of Akt1 and decreased expression of Akt2 and PTEN (131). *In vitro*, IL-12 directly activates PI3K/Akt/Foxo pathway to drive Th1 polarization in Treg cells (131). Moreover, Foxo1 can be recruited to a regulatory element upstream of the transcriptional start site of *Ifng* gene. Treg cell-specific deletion of Foxo1 leads to upregulation of *Ifng* gene expression and increased IFNγ$^+$ Treg cells (95, 96). Notably, the suppressive activity of Foxo1-deficient Treg cells is mostly corrected by blockade of PI3K/Akt pathways (95). As described above, decreased Foxo activity is correlated with increased glycolysis in T cells (98), and increased glycolysis is recently reported to promote Th1 cell differentiation by epigenetic regulation of *Ifng* gene locus (138), suggesting that Treg cells may also adopt similar interplay between metabolic and epigenetic regulation to polarize Th1-like differentiation. In further support of this notion, a recent study has reported that deficiency of the E3 ubiquitin ligase VHL causes Treg cells to

adopt an IFN-γ^+ Th1-like Treg cell program (139). This event is mediated through both a shift in glycolytic programming driven by HIF-1α, and increased binding of HIF-1α to the *Ifng* gene, further strengthening the link between glycolysis and IFN-γ^+ Treg cell generation. Whether metabolites or key glycolytic enzymes promote the generation of Th1-like Treg cells needs further investigation.

For Th2-like Treg cells, both aberrant and beneficial phenotypes have been observed. Toward the former, programming of Th2-like Treg cells is associated with compromised suppressive function in respiratory syncytial virus (RSV) infection and food allergy mouse models (140, 141). Treg cell-specific deletion of E3 ubiquitin ligase Itch also leads to generation of Th2-like Treg cells that can drive Th2-related pathologies (142). As noted above, Th2-like Treg cells are characterized by expression of Gata3 and IRF4, as well as production of IL-4 and IL-13 (140–142). Th2-like programming can be induced by IL-4R signals that promote Gata3 expression (140, 141). Additionally, TCR signals induce IRF4 expression in Treg cells, and mice with Treg cell-specific deletion of IRF4 develop type-2 inflammation, as IRF4 expression suppresses expression of pro-inflammatory Th2-specific genes in Treg cells (119). IRF4 can also form complex with Foxp3 to regulate the transcriptional program and generation of effector Treg (eTreg, $CD44^+CD62L^-$) cells (119, 143). mTOR functions downstream of TCR to drive IRF4 expression to control Th2 inflammation (85). Moreover, defects in metabolic reprogramming caused by LKB1 deletion in Treg cells are associated with impaired suppression of Th2 immunity (113). These results together suggest that metabolic pathways may have a great impact upon Th2-like Treg cells, although the precise mechanisms have not been defined.

Th17-like Treg cells co-express RORγt with Foxp3 (144, 145) and can be generated in the periphery (145). Also, under inflammatory conditions, RORγt$^+$ Treg cells can be generated from tTreg cells (146). These IL-17-producing Treg cells retain suppressive function (144, 145, 147). IL-6 and downstream STAT3 activation, as well as IL-23, IL-1β, and IL-21, are critical for the generation of IL-17-producing Treg cells (148–150). The tissue in which Th17-like Treg cells have been best characterized is the intestine, where Treg cells adapt to the local environmental changes through functional and phenotypic reprogramming as discussed more below. In addition, as a central coordinator of metabolism in T cells, mTOR also controls Treg cell polarization into other functionally plastic subpopulations, such as Tfr cells in the germinal center (137). Mechanistically, mTORC1 promotes the phosphorylation of the transcription factor STAT3 and induces the expression of the transcription factor TCF-1 to drive Bcl-6 expression. These results together indicate that metabolism plays a critical role in modulating Treg cell plasticity. However, the limitations of low cell numbers and inability to isolate Th-like Treg cells based on surface markers impede analysis of how metabolism controls their homeostasis and function. The development of single cell metabolomics (151, 152) and new fate-mapping genetic tools (118, 125) could help understand the metabolic regulation of Th-like Treg cells in the future.

METABOLIC ADAPTION IN TISSUE TREG CELLS

An emerging concept is that Treg cells found in non-lymphoid organs, including adipose tissue, intestine, and tumors, display marked tissue-specific heterogeneity. Tissue-specific transcriptional programs, possibly orchestrated by unique transcription factors, such as PPARγ, RORγt, and Gata3, facilitate the ability of tissue-resident Treg cells to maintain tissue homeostasis. Non-lymphoid and tumor tissues have dramatic differences in their metabolic environments than secondary lymphoid organs, thus suggesting differences in their nutrient and intracellular metabolic requirements. Notably, Treg cells in non-lymphoid organs and tumor tissues are largely eTreg cells. mTOR has been identified as a critical driver for the generation and function of eTreg cells in both lymphoid and non-lymphoid tissues (85, 153), which acts downstream of TCR signals to drive IRF4 expression and mitochondrial metabolism, facilitating eTreg cell generation. The result further underscores a central role of metabolism in modulating tissue Treg cell heterogeneity and homeostasis (**Figure 3**).

Adipose Tissue

Among different types of adipose tissues, visceral adipose tissue (VAT) is enriched for Treg cells (hereafter called VAT-Treg cells) whose major nutrient source seems to be fatty acids derived from the environment. VAT-Treg cells display a distinct gene expression signature compared to Treg cells from secondary lymphoid organs (154). Peroxisome proliferator-activated receptor gamma (PPARγ) expression is critical for establishing the unique VAT-Treg cell transcriptional program and homeostasis (155). Indeed, mice that specifically lack PPARγ in Treg cells have a significantly reduced population of VAT-Treg cells, but not Treg cells in other organs. The increased expression of PPARγ promotes fatty acid metabolism and stimulates the accumulation and suppressive activities of the VAT-Treg cells (155). Moreover, VAT-Treg cells express CD36, a receptor that facilitates uptake of long-chain fatty acids and contributes to lipid accumulation in contexts of high-fat diets and obesity (155). Obese mice display significantly decreased VAT-Treg cells, which may be partly explained by the heightened leptin signaling (108, 156). Leptin may be produced by adipose cells (157). VAT-Treg cells express leptin receptor, receive increased leptin secretion from VAT of obese mice and decrease their proliferation and number, suggesting that leptin may be an important environmental cue in the adipose tissue for modulating Treg cells (21). This observation is consistent with other studies that identified a central role of leptin in inhibiting Treg cell proliferation and resulting in Treg cell anergy *in vitro* (108, 156). In addition, a recent report has found that the enzyme hydroxyprostaglandin dehydrogenase (HPGD) is highly expressed in VAT-Treg cells, which is dependent on PPARγ. HPGD catabolizes prostaglandin E_2 (PGE_2) into the metabolite 15-keto PGE_2 to suppress conventional T cell activation and proliferation (158). These results together demonstrate that Treg cells adapt to unique VAT environments for their homeostasis and function.

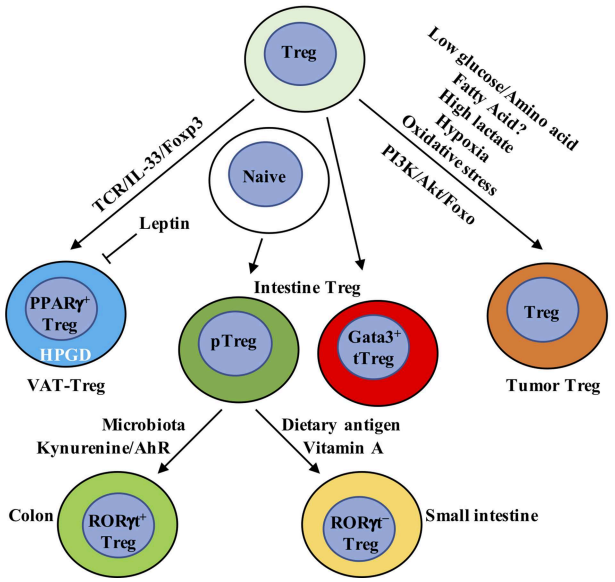

FIGURE 3 | Metabolic and transcriptional adaption in tissue Treg cells. VAT-Treg cell accumulation is dependent upon TCR, Foxp3, and IL-33 signaling. VAT macrophages and DCs are the major antigen-presenting cells that express MHC-II to activate and increase VAT-Treg cells. In VAT, white adipose tissue produces IL-33 to increase VAT-Treg cell generation. Leptin produced by adipose cells inhibits VAT-Treg cell proliferation and induces anergy. Leptin-neutralizing antibody reverses anergy and increases mTOR activation and Treg cell proliferation. VAT-Treg cells have high expression of enzyme hydroxyprostaglandin dehydrogenase (HPGD), which is dependent on PPARγ. HPGD produces 15-keto PGE_2 to suppress conventional T cell activation and proliferation. Intestinal Treg cells mainly contain Gata3$^+$ tTreg, RORγt$^+$, and RORγt$^-$ pTreg cells. RORγt$^+$ pTreg cells are the dominant population in the colon, while RORγt$^-$ pTreg cells mainly localize in small intestine. Their difference is mainly derived from the dominant effect of microbiota and dietary metabolites, respectively, in the colon and small intestine. In the colon, metabolism of tryptophan produces AhR ligands, such as kynurenine, to selectively enhance the generation of Foxp3$^+$ Treg cells. In the small intestine, dietary vitamin A is present at high concentrations. RA, a metabolite from vitamin A, induces pTreg cells from naïve T cells in the small intestine in combination with TGF-β. RA also induces the expression of CCR9 on the surface of Treg cells to facilitate their migration to the small intestine. In tumor tissue, Treg cells not only display eTreg cell phenotypes similar to non-lymphoid organs but also express some tumor-specific gene signatures. Compared to conventional T cells, Treg cells have a metabolic advantage in tumor microenvironment, which has low glucose/amino acid, high lactate/hypoxia, and elevated oxidative stress. Genetic or pharmacological perturbation of metabolic pathway PI3K/Akt/Foxo signaling directly impacts tumor-resident Treg cell homeostasis, function and anti-tumor immunotherapy. VAT, visceral adipose tissue; AhR, aryl hydrocarbon receptor.

Recently, using a T cell receptor transgenic mouse line in which VAT-Treg cells are enriched, the Mathis group identified a two-step, two-site developmental axis for VAT Treg cells (159). Single cell RNA-sequencing (scRNA-seq) and assay for transposase-accessible chromatin sequencing (ATAC-seq) analyses reveal that splenic Treg cells that are destined to become VAT-Treg are primed within the secondary lymphoid organs, where they are defined by low expression of PPARγ that allows them to upregulate selective VAT-Treg-associated transcriptional programs. These PPARγ^{lo} Treg cells then migrate to the VAT, where they are educated and exposed to local microenvironmental cues to differentiate into VAT-Treg cells. The accumulation of VAT-Treg cells is dependent upon TCR, Foxp3, and IL-33 signaling, and is associated with substantial epigenetic remodeling. Specifically, the critical role of IL-33 in the development and maintenance of VAT-Treg cells is dependent upon the high expression of IL-33 receptor ST2 (160). IL-33 treatment leads to reduced obesity, improved glucose tolerance, and increased proportion of ST2$^+$ Treg cells in the VAT of genetically obese diabetic mice (161). White adipose tissue is the main source of IL-33 in VAT (162), and γδ T cells may also promote IL-33 production by VAT, as mice lacking γδ T cells exhibit decreases of ST2$^+$ Treg cells in VAT (163). Subsequent studies also demonstrate that IL-33 is a critical driver for the differentiation and function of other tissue-resident Treg cells, including in the brain (164) and intestine (165). Moreover, TCR: MHC-II interactions are also required for the development and maintenance of VAT-Treg cells (166). In MHC-II-deficient mice, VAT-Treg cell number is greatly reduced, associated with less expression of Gata3 (160). Among the antigen-presenting cells that express MHC-II, VAT macrophages (167) and DCs (160) have been reported to activate and increase VAT-Treg cells. These results together demonstrate that the ligand-receptor interactions between cells in the adipose tissues instruct plasticity in the VAT and generate unique VAT-Treg cells. However, questions regarding the phenotype and function of VAT-Treg cells in humans and how to manipulate VAT-Treg cells to prevent obesity and related metabolic diseases still remain to be addressed.

Intestine

The intestine is constituted by small intestine and large intestine, with the latter containing cecum and colon. Due to the direct exposure to exogenous dietary antigens, the intestine must both protect the host from harmful pathogens and maintain tolerance to intestinal microbiota and beneficial metabolites. This sophisticated task requires a close collaboration within the intestinal network to recognize and distinguish the harmful and beneficial material from the diet. The intestinal wall contains four major layers: mucosa, submucosa, muscularis, and serosa (168). The epithelial cells of the mucosa form the first physical barrier and are supported by an underlying network of immune cells in the lamina propria, local lymphoid structure like Peyer's patches (PPs) and the draining lymph nodes. Treg cells play a critical role in suppressing local inflammatory responses and maintaining intestinal homeostasis (169). To adapt to the highly specific environment, Treg cells develop tissue-specific heterogeneity. Indeed, a substantial proportion of Helios$^-$neuropilin-1$^-$ pTreg cells are found in the small intestine and colon, as well as Helios$^+$neuropilin-1$^+$ tTreg cells that express Gata3 (170). Thus, the intestine represents a unique mucosal site that requires both tTreg and pTreg cells for homeostasis.

The cues that allow for pTreg cell induction in the intestine have been extensively studied. Due to the similar differentiation requirements and expression of RORγt, RORγt$^+$Foxp3$^+$ Treg cells were once thought to be precursors for unstable Treg cells that would differentiate into Th17 cells (171, 172). However, a large population of RORγt$^+$ pTreg cells are present in the

colon under steady state (170, 173, 174), while the small intestine mainly contains RORγt$^-$ pTreg cells (175). Transcriptomic analysis has revealed that the signature of RORγt$^+$Foxp3$^+$ Treg cells is similar to both Treg and Th17 cells, but has a higher overlap with Treg cells (174). Further, RORγt$^+$Foxp3$^+$ Treg cells display significantly demethylation at Treg cell-specific signature genes like *Foxp3*, *Ctla4,* and *Tnfrsf18*, indicating that they are a lineage-stable population with potent suppressive activity (174). TCR repertoire analysis further reveals that the RORγt$^+$Foxp3$^+$ population is distinct from other colonic T cell subsets (176). The development of RORγt$^+$Foxp3$^+$ cells in the mucosa can be derived from naïve CD4$^+$ T cells in the periphery and pass through a RORγt$^-$ Treg intermediate before co-expressing RORγt (176). CX3CR1$^+$ antigen-presenting cells may provide specific signals for the development of RORγ$^+$ Treg cells (176). Moreover, loss of RORγt$^+$Foxp3$^+$ Treg cells exaggerates several models of mucosal autoimmunity, indicating that this subpopulation is functionally important (170, 173, 174). Thus, co-expression of RORγt and Foxp3 is also essential for establishing Treg cell function in the intestine.

The intestinal microenvironment differs in the colon and small intestine. The colon harbors multiple species of microbiota to facilitate the development of RORγt$^+$ Treg cells (170, 173). Germ-free mice treated with antibiotics greatly diminish the abundance of colonic pTreg cells, leaving a predominant Helios$^+$ tTreg cell population (173, 177, 178). The abundance of RORγt$^+$ Treg cells is linked to changes in the microbiota, whereas RORγt$^-$ Treg cells are relatively insensitive (170, 173, 175, 179). Many microbial species such as *Clostridium ramosum, Staphylococcus saprophyticus, Bacteroides thetaiotaomicron,* and *Clostridium histolyticum,* have the potential to induce pTreg cell generation (170, 173, 180–183). In addition, *Helicobacter hepaticus*-reactive naïve CD4$^+$ T cells differentiate into RORγt$^+$ Treg cells upon *H. hepaticus* colonization, which is dependent upon the transcription factor c-Maf (183). The RORγt$^+$ Treg cell differentiation is also dependent upon constant antigen exposure, as mice deficient in MHC-II have greatly fewer RORγt$^+$ Treg cells (170). The aryl hydrocarbon receptor (AhR) is a sensor that detects environmental pollutants and physiological compounds from diet, host cells, and microbiota (184). Microbiota, such as *Lactobacillus* species, metabolize tryptophan to generate AhR ligands (185). Metabolism of tryptophan produces a series of AhR ligands, such as kynurenine, to selectively enhance the generation of Foxp3$^+$ Treg cells *in vitro* (186). Intriguingly, the microbiota-induced RORγt$^+$ Treg cells express the highest amount of AhR compared to various organs (179). Treg cell-specific deletion of AhR impairs pTreg, but not tTreg, cell homeostasis in the colon (179). Moreover, AhR-expressing Treg cells have enhanced *in vivo* suppressive activity compared with Treg cells lacking AhR expression in a T cell transfer model of colitis, highlighting a central role of AhR in microbiota-induced pTreg cell generation and function (179). Microbiota also increase the expression of GPR15, an orphan G-protein coupled receptor, to promote the specific homing of Treg cells to the large intestine (187). Therefore, microbiota promote the generation, function, and influx of Treg cells in the large intestine.

Studies comparing changes in dietary composition and germ-free mice indicate that the induction of Treg cells in the small intestine is mainly dependent upon dietary antigens rather than microbiota, and diet-generated Treg cells are mostly pTreg cells (175, 182, 188). For example, all-*trans* RA acts in concert with TGF-β for pTreg cell generation in the small intestine (53, 189, 190), where dietary vitamin A is abundant (191) and is converted to RA with the help of local epithelial cells and DCs (192). Moreover, RA induces the expression of CCR9 on Treg cells, which is required for their migration to small intestine (53, 189). RA-induced pTreg cell generation underlies oral tolerance, as mice treated with a vitamin A-deficient diet display defective in the induction of oral tolerance (193). Recent studies have also indicated a critical role for isoleucine in regulating Treg cell homeostasis in the small intestine (194). Other dietary mechanisms controlling the homeostasis of small intestinal Treg cells *in vivo* require further investigation.

Tumor Tissue

Tumor tissues are complex environments that contains tumor cells, stromal cells, and infiltrating immune cells (195–197). Due to the malignant cell proliferation, tumor cells require sufficient energy supply and exert metabolic effects on local tumor microenvironment (198). A large population of Treg cells exist in the tumor microenvironment and present a major obstacle for effective anti-tumor therapy (195, 196). Treg cells isolated from tumors are often in an activated state with a metabolic signature that is distinct from lymphoid tissue Treg cells. Their transcriptome shares high similarities with "tissue Treg cells" residing in non-lymphoid tissues (199). Recent studies have identified an important role of Foxo1 in regulating eTreg cells in tumor (200). Hyperactivation of Foxo1 preferentially depletes eTreg cells, which enhances CD8$^+$ T cell function and anti-tumor immunity (200), suggesting that targeting PI3K/Akt/Foxo pathway in Treg cells could possibly break the Treg cell barrier in anti-tumor therapy. Inactivation of PI3K p110δ in Treg cells also unleashes cytotoxic CD8$^+$ T cells and induces tumor regression and better survival (201–203). These data reveal that subtle perturbations in metabolic signaling could impact tumor-resident Treg cell homeostasis and function. Despite the similarities with Treg cells in non-lymphoid organs, tumor-resident Treg cells also highly express unique signatures, such as *Ccr8*, *Tnfrsf8, Cxcr3*, and *Samsn1* (199, 204), which might serve as valuable targets for tumor immunotherapy.

Tumor cells modulate several environmental cues to affect tumor-resident Treg cell generation and function, in which the competition of extracellular nutrients stands out. Cancer cells undergo a transition of OXPHOS to aerobic glycolysis, known as the Warburg effect (198). This metabolic shift in cancer cells leads to the consumption of environmental glucose and glutamine, thus leading local T cells to adopt unresponsive or functionally exhausted states (205, 206). In contrast, glucose deprivation can drive Foxp3 expression, shifting T cell differentiation from conventional T cells toward iTreg cells (16, 78, 207). Likewise, tumor cells also compete with T cells for extracellular amino acids for metabolism. Amino acids, especially glutamine and leucine, are required to fuel mTORC1 activation in conventional

T cells, promoting T cell differentiation toward Th1, Th2, and Th17 cells, but have a less effect on iTreg cells (208). Deletion of amino acid transporters ASCT2 and SLC7a5 (LAT1) leads to normal Treg cell differentiation (209, 210). Moreover, a decrease of intracellular αKG, caused by the limited availability of extracellular glutamine, also promotes the generation of iTreg cells rather than Th1 cells (211). Interestingly, Treg cells could further deplete environmental amino acid levels by inducing amino acid-consuming enzymes in DCs, such as arginase 1, histidine decarboxylase, or threonine dehydrogenase, through TGF-β and IL-10 secretion. Enzymatic consumption of amino acids by DCs could further inhibit mTORC1 activation, thus synergizing with TGF-β for Treg cell induction (212). These results indicate that nutrient availability in the tumor microenvironment can suppress T cell responses, in part, through induction of Treg cells.

Apart from glucose and amino acids, tumor cells also perturb environmental fatty acid concentrations. In some cancer cells, the extracellular liberation of free fatty acids from more complex lipid species is increased (198). However, the exact regulation of fatty acid availability and diversity in the tumor microenvironment remains elusive and debatable. Mouse iTreg cells are reported to preferentially use FAO (16), and short chain fatty acids can promote iTreg cell differentiation (88, 213). In brain tumors, higher percentages of Treg cells that express CD36 and SLC27A1 (fatty acid transporters) are found (83). Inhibition of lipid uptake with sulfo-N-succinimidyl oleate (SSO) or FAO with etomoxir (Eto) prevents Treg cell immunosuppressive capabilities in this environment (83). Therefore, future studies could analyze if Cpt1a deficiency affects specific tumor Treg cell responses (91). Under steady state, Treg cells rely on mTORC1-mediated lipogenesis for their expansion and function (22), and proliferating Treg cells in the MC38 tumor microenvironment also rely on *de novo* fatty acid synthesis to accumulate intracellular lipids and complement glycolysis for Treg cell expansion (214). Therefore, whether nutrient competitiveness influences and whether fatty acids are preferentially catabolized or synthesized to support Treg cell accumulation or function in tumors await detailed investigation.

Metabolic factors other than nutrients also contribute to heightened Treg cell accumulation and function in the tumor microenvironment. Hypoxia is common in some regions of tumor tissue due to lack of vascularity (215). HIF-1α is usually upregulated upon hypoxia, and is positively correlated with the malignancy of certain tumors (216). Previous studies have identified HIF-1α as a negative regulator of iTreg cell differentiation, while it promotes Th17 cell differentiation (78, 217). However, HIF-1α is required for optimal Treg cell suppressive activity *in vivo* (218). A study using mice with Treg cell-specific deletion of HIF-1α has revealed that, under hypoxia in a mouse model of glioma, HIF-1α directs glucose away from the mitochondria, leaving Treg cells dependent on fatty acids for mitochondrial metabolism (83). This mechanism provides an advantage for Treg cells to thrive in low-glucose tumor microenvironment. The tumor microenvironment also accumulates excess lactate produced by tumor cells due to their high rates of glycolysis (198). A major consequence of lactate secretion is microenvironmental acidification (198). Multiple studies have identified a deleterious role of lactate in inhibiting $CD8^+$ T cell-mediated anti-tumor immunity, while neutralization of acidosis improves checkpoint blockade (219–221). In contrast, Treg cell suppressive function and proliferation are unaffected by the addition of lactate due to reduced glycolysis (17). Mechanistically, conventional effector T cells balance NAD^+/NADH ratios through conversion of pyruvate into lactate to maintain glycolysis (17, 138), whereas Treg cells do not require high rates of glycolysis and can thus balance NAD^+/NADH through oxidation of exogenous lactate (17). As a result, loss of glycolysis is detrimental to conventional effector T cells but not Treg cells (16), providing Treg cells with a metabolic advantage in response to low-glucose and high-lactate tumor microenvironment (17). Furthermore, oxidative stress is an additional metabolic feature in the tumor microenvironment (198). Treg cells are relatively more sensitive to oxidative stress than conventional T cells and undergo potent apoptosis in the tumor microenvironment (222). Intriguingly, apoptotic Treg cells have been revealed to mediate superior immunosuppression through converting a large amount of ATP to adenosine via CD39 and CD73 (222), suggesting that tumor-resident Treg cells sustain and amplify suppressive activity by inadvertent death via oxidative stress. These observations all underscore the importance of metabolic adaption of Treg cells in tumor microenvironment for their suppressive activity, although the precise metabolic status of tumor Treg cells needs to be determined, such as by single cell metabolomics (151) or computational-based inference of metabolic gene expression in scRNA-seq data (223).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Current studies illustrate that environmental and intracellular metabolic factors orchestrate Treg cell stability, plasticity, and tissue-specific heterogeneity in different contexts. How Treg cells metabolically adapt to different environmental contexts *in vivo* still remains underexplored. From the perspective of intricate molecular network, mTOR likely acts as a "metabolic hub" that senses and adjusts the cellular metabolic state, thereby balancing the catabolism and anabolism in Treg cells to define their functional and phenotypic state. Therefore, dissecting the upstream metabolic inputs to mTOR in Treg cells remains a key future direction and may uncover how environmental cues are transmitted to Treg cells for functional reprogramming. Apart from mTOR, the roles of other metabolic components, such as mitochondrial respiratory chain complexes, are being uncovered in Treg cells. It will be intriguing to determine whether the loss of various metabolic components within Treg cells causes distinct pathologies in mice. Utilizing CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) screening, novel metabolic regulators in Treg cell stability, plasticity and tissue-specific heterogeneity may be discovered, to further expand our view on Treg cell-specific metabolic regulation.

Unlike Treg cell instability, few studies have discussed the role of metabolism for Th-like Treg cell generation. The transcriptional landscape could be altered in Th-like Treg cells to allow the co-expression of Th transcription factors and inflammatory cytokines, along with Foxp3 expression, which may involve epigenetic regulation, such as histone acetylation. Metabolites such as acetyl-CoA can directly alter the activity of chromatin-modifying enzymes, including histone acetyl-transferases (HATs), histone methyl-transferases (HMTs) and sirtuins. It will be interesting to uncover the metabolic pathways and their key drivers that orchestrate the epigenetic landscape of the Th transcription factors and inflammatory cytokines in Th-like Treg cells. The development of ATAC-seq has allowed analysis of transcription factor occupancy in specific cell types, which provides a starting point for studying the interplay between metabolism and epigenetics in shaping Treg cell plasticity (224).

Technological advances, such as scRNA-seq, are likely to uncover how Treg cells metabolically adapt to different environments. For instance, a recent scRNA-seq analysis of colonic and skin Treg cells revealed that Treg cells undergo metabolic reprogramming as they migrate from lymphoid organ to non-lymphoid barrier organs (225). Specifically, Treg cells convert from expressing glycolysis- and migration-associated genes toward expressing genes associated with fatty-acid metabolism and cytokine production. Intriguingly, lymphoid organs are glucose-rich relative to non-lymphoid organs or tumor microenvironment (169), while non-lymphoid organs may express higher levels of fatty acids. These differences in local nutrient composition likely explain why Treg cells from adipose tissue and intestine express higher levels of the fatty acid-binding transporters than those in lymphoid tissues. The interplay between local nutrient composition and Treg cell stability, plasticity, and tissue-specific heterogeneity also likely accounts for the unique physiological functions of Treg cells in non-lymphoid organs, such as controlling adipose tissue homeostasis (226). As scRNA-seq, ATAC-seq, *in situ* imaging, and metabolic profiling have greatly expanded the possible view of cell-cell communication (227, 228), it may be possible to use these technologies to discover novel ligand-receptor pairs or signaling pathways responsible for Treg cell stability and plasticity in these sites. Recent work has also established that Treg cells from hypoxic tumors express higher fatty acid transporters and catabolize free fatty acids for immunosuppression (83). Thus, understanding the detailed mechanisms of metabolic cross-talk between Treg cells and tumor cells may uncover essential regulatory networks in tumorigenesis, tumor progression, and therapy resistance. Therefore, analysis of Treg cell-metabolic interactions may provide new opportunities to develop novel Treg cell-based immune-metabolic interventions for the treatment of inflammatory diseases and tumor progression.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Immune Checkpoints in Circulating and Tumor-Infiltrating CD4$^+$ T Cell Subsets in Colorectal Cancer Patients

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Blockade of inhibitory immune checkpoints (ICs) is a promising therapeutic approach; however, it has shown limited success in some cancers including colorectal cancer (CRC). The tumor microenvironment (TME) is largely responsible for response to therapy, and its constituents may provide robust biomarkers for successful immunotherapeutic approaches. In this study, we performed phenotypical characterization and critical analyses of key inhibitory ICs and T regulatory cell (Treg)-related markers on CD4$^+$ T cell subsets in CRC patients, and compared with normal colon tissues and peripheral blood from the same patients. We also investigated correlations between the levels of different CD4$^+$ T cell subsets and the clinicopathologic features including disease stage and tumor budding. We found a significant increase in the levels of CD4$^+$FoxP3$^+$Helios$^+$ T cells, which represent potentially highly immunosuppressive Tregs, in the CRC TME. Additionally, tumor-infiltrating CD4$^+$ T cells upregulated programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), T cell immunoglobulin and mucin domain-3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3). We also characterized the expression of PD-1, CTLA-4, TIM-3, and LAG-3 on different CD4$^+$FoxP3$^{-/+}$Helios$^{-/+}$ T cell subsets. Interestingly, we found that CTLA-4, TIM-3, and LAG-3 were mainly co-expressed on FoxP3$^+$Helios$^+$ Tregs in the TME. Additionally, FoxP3^{high} Tregs expressed higher levels of Helios, CTLA-4 and TIM-3 than FoxP3^{low} T cells. These results highlight the significance of Tregs in the CRC TME and suggest that Tregs may hamper response to IC blockade in CRC patients, but effects of different IC inhibition regimes on Treg levels or activity warrants further investigations. We also found that CD4$^+$CTLA-4$^+$ T cells in circulation are increased in patients with advanced disease stage. This study simultaneously provides important insights into the differential levels of CD4$^+$ T cell subpopulations and IC expression in CRC TME, compared to periphery and associations with clinicopathologic features, which could be used as potential biomarkers for CRC progression and response to therapy.

Keywords: colorectal cancer, T regulatory cells, immune checkpoints, tumor microenvironment, T cells

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INTRODUCTION

The tumor immune microenvironment (TIME) is largely accountable for response to immunotherapeutic modalities, and better analyses of its constituents can help develop robust biomarkers to identify patients who would respond to immunotherapy (1). In addition, DNA fragments or tumor cells budded off from the primary tumor sites may also be detected in "liquid biopsies" and used as potential biomarkers for initiation of effective anti-tumor therapies (2, 3).

Colorectal cancer (CRC) is among the leading causes of cancer-related mortality and morbidity worldwide, affecting ∼1.4 million newly diagnosed patients and causing death in 0.7 million every year (4, 5). Current treatments for primary and metastatic CRC primarily include laparoscopic surgeries, radiotherapy, and neoadjuvant and palliative chemotherapies (6, 7). Immunotherapy regimes however have not had a big impact on treating CRC as in treating other malignancies. Nonetheless, pembrolizumab, an immune checkpoint (IC) inhibitor [anti-programmed cell death protein 1 (PD-1)], was recently granted Food and Drug Authority (FDA) approval for treating unresectable or metastatic solid tumors, including CRC, with microsatellite instability high (MSI-H) or DNA mismatch repair deficiency (dMMR) (8, 9).

Inhibitory ICs attenuate T cell responses to mediate immune tolerance (10). These immune-inhibitory pathways are often employed by tumors to facilitate immune evasion. High Treg infiltration coupled with high IC expression in the tumor microenvironment (TME) should further promote tumor progression due to T cell exhaustion and impaired cytokine release. Several studies have reported accumulation of highly suppressive Treg populations and elevated IC expression in the colorectal TME (11–13). However, accumulation of Tregs in CRC patients can have opposing effects on prognosis also as it may be associated with favorable clinical outcomes (14, 15).

In this study, we investigated the immune landscape of colorectal tumors, compared to normal colon tissues and peripheral blood from the same patients. We focused our investigations on $CD4^+$ T cells and on the expression of key inhibitory ICs and regulatory T cell (Treg)-related markers. Tumor-specific T cells are a key component of the TME due to the presence of a multitude of suppressive mechanisms within the TME, which assist tumor immune evasion. Accumulation of Tregs within the TME leads to an immune-permissive microenvironment, favoring uncontrolled tumor growth (16, 17). Potent anti-tumor immune responses require a shift in balance between levels of Tregs and T effector cells (Teff) in the TME (18). Therefore, T cell trafficking and localization into tumor sites and preferential proliferation and differentiation of tumor-reactive T cells can facilitate effective immunotherapies (19). Moreover, T cell inflamed tumors, characterized by existing anti-tumor T cell responses, are associated with improved clinical outcomes in CRC patients (11, 20).

We found a significant increase in $CD4^+$ T cells in the CRC TME, compared with adjacent normal tissue. Moreover, these $CD4^+$ T cells comprised of potentially suppressive $FoxP3^{high}$ Treg populations, which co-expressed high levels of Helios, previously reported as a marker for activated Tregs (21). Additionally, we found that intratumoral $CD4^+$ T cells upregulate multiple inhibitory ICs including PD-1, cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), T cell immunoglobulin and mucin domain-3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3). We also compared the levels of different $CD4^+$ T cell subsets between CRC patients presenting with early and advanced stage disease, and between patients who showed varying tumor budding status. We found that patients with advanced stage disease have increased CTLA-4 expression on $CD4^+$ T cells in circulation. Overall, this study increases our knowledge about the potential use of checkpoint blockade in CRC patients.

MATERIALS AND METHODS

Sample Collection and Storage

This study was performed under ethical approvals from Qatar Biomedical Research Institute, Doha, Qatar (Protocol no. 2018-018) and Hamad Medical Corporation, Doha, Qatar (Protocol no. MRC-02-18-012). All experiments were performed in accordance with relevant guidelines and regulations.

Peripheral blood samples were collected in EDTA tubes from 34 CRC patients, and tumor tissues (TT) and paired, adjacent non-cancerous normal colon tissues (NT) were obtained from 27 out of these 34 patients, who underwent surgery at Hamad Medical Corporation, Doha, Qatar. All patients included in the study were treatment-naïve prior to surgery and provided written informed consent prior to sample collection. **Table 1** shows the clinical and pathological characteristics of the study population.

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood by density-gradient centrifugation using

TABLE 1 | Characteristic features of study populations.

	CRC patients
Number	34 (27)†
Age (median)	62 (31-96)§
Gender (Male: Female)	24:10
TNM stage	
I	6 (1)†
II	10 (10)†
III	15 (13)†
IV	3 (3)†
DNA mismatch repair deficiency (dMMR)	4 (3)†
Tumor budding	
Low	13 (11)†
Intermediate	11 (7)†
High	10 (9)†
Histological grade	
G2 Moderately differentiated	All samples

CRC; Colorectal cancer.
§Median range.
†Samples used for analyses of tumor-infiltrating immune cells.

Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). PBMC were frozen in freezing media (50% FBS, 40% RPMI 1640 media and 10% DMSO) at a density of 5 million cells per 1 ml in cryovials to be used in batches for subsequent analyses. Tissue specimens were also stored in freezing media for subsequent analyses.

Cell Isolation From Colorectal Tumors and Normal Colon Tissues

Cells were isolated from NT and TT by mechanical disaggregation. Briefly, tissues frozen in freezing media were thawed and washed with phosphate-buffered saline (PBS) and then mechanically cut into small pieces (∼2–4 mm) using a surgical scalpel. Tissue disaggregation was performed on a gentleMACS dissociator (Miltenyi Biotech, Bergisch Gladbach, Germany) without using enzymes. The cell suspension was then passed through a 100 μM cell strainer to remove aggregates and debris. The single cell suspension was washed with PBS and stained for flow cytometric analyses.

Multi-Parametric Flow Cytometry

PBMC and cells isolated from tissues were washed with PBS and re-suspended in 100 μl flow cytometry staining buffer (PBS with 1% FCS and 0.1% sodium azide). Fc receptors (FcR) were first blocked using FcR Blocker (Miltenyi Biotec). Fixable Viability Dye eFluor 780 (eBioscience, San Diego, USA) was added to gate live cells. Cells were then stained with cell surface antibodies including CD3-Alexa Fluor 700 (clone UCHT-1; BD Biosciences, Oxford, UK), CD4-phycoerythrin (clone RPA-T4; BD Biosciences), CD25-Brilliant Violet 650 (clone BC96; BioLegend, San Diego, USA), PD-1-PE/Dazzle™ 594 (clone EH12.2H7; BioLegend), LAG-3-Brilliant violet 421 (clone T47-530; BD Biosciences) and TIM-3-Brilliant Violet 711 (clone 7D3; BD Biosciences) and incubated at 4°C for 30 min. Cells were then washed twice with flow cytometry staining buffer. For intracellular staining, cells were incubated at 4°C for 45 min in fixation/permeabilization buffer (eBioscience). Cells were then washed twice with permeabilization wash buffer (eBioscience). Mouse serum (Sigma-Aldrich) and rat serum (Sigma-Aldrich) were added to block non-specific binding sites for 10 min at 4°C. Intracellular antibodies including CTLA-4-PerCP-eFluor 710 (clone 14D3; eBioscience), FoxP3-phycoerythrin cyanin 7 (PE/Cy7) (clone PCH101; eBioscience) and Helios-Fluorescein Isothiocyanate (FITC) (clone 22F6; BioLegend) were then added and cells incubated for another 30 min at 4°C. Cells were then washed twice with permeabilization wash buffer (eBioscience), and re-suspended in flow cytometry staining buffer.

All data were acquired on a BD LSRFortessa X-20 SORP flow cytometer using BD FACSDiva software (BD Biosciences) and analyzed on FlowJo V10 software (FlowJo, Ashland, USA).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, California, USA). One-way Anova test was performed to check for statistical significance in grouped analyses. Paired *t*-tests were performed within groups on samples that passed the Shapiro-Wilk normality test, while Wilcoxon matched-pairs signed rank tests were performed on samples that did not show normal distribution. Unpaired *t*-tests were performed for comparisons between groups on normally distributed data and Mann-Whitney tests for samples that did not show normal distribution. A $P > 0.05$ was considered statistically non-significant. The *P*-values are represented as follows; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

Increased Levels of CD4+ T Cells in the Tumor Microenvironment of Colorectal Cancer Patients

Accumulation of tumor-infiltrating T cells in CRC patients has been previously reported, and shown to be associated with favored clinical outcomes (11). We investigated the levels of $CD4^+$ T cells in circulation, normal colon tissues and in the TME of CRC patients. The overall levels of circulating $CD4^+$ and $CD4^-$ T cells in our cohort were similar ($CD4^+$; 31.4% vs. $CD4^-$; 31.7%, **Figure 1A**). In agreement with previous reports, we found that $CD4^+$ T cells accumulate in colorectal tumors, compared with normal tissues but were lower compared to their levels in circulation (PBMC; 31.4 ± 2.0 vs. NILs; 11.5 ± 1.0 vs. TILs; 22.0 ± 2.1, **Figure 1A**). Levels of Teff cells within the TME can greatly affect cancer progression and response to therapy (18). Therefore, we focused our subsequent investigations to perform further phenotypical characterization of $CD4^+$ T cell subsets to ascertain their role in colorectal tumor biology.

Tumor-Infiltrating CD4+ T Cells in CRC Patients Comprise Mainly of Potentially Suppressive T Regulatory Cells

Tregs constitute an important subset of $CD4^+$ T cells, which are characterized by high expression of interleukin-2 receptor alpha chain (CD25) and forkhead box P3 (FoxP3) transcription factor (22). Moreover, Helios is a key transcription factor, which regulates $FoxP3^+$ Treg functional stability and it is required for their inhibitory activity (23). Infiltration of $FoxP3^+$ Tregs is often associated with poor prognosis and disease progression (24). We found that the levels of $CD4^+CD25^+$, $CD4^+FoxP3^+$ and $CD4^+Helios^+$ T cells were significantly higher in the TME, compared with NT and circulation (CD25: PBMC; 5.0 ± 0.6 vs. NILs; 2.6 ± 0.4 vs. TILs; 13.0 ± 1.8, FoxP3: 6.5 ± 0.7 vs. 9.8 ± 0.8 vs. 26.8 ± 2.8 & Helios: 9.1 ± 0.8 vs. 11.8 ± 1.0 vs. 23.1 ± 2.5, **Figures 1B–D**). We also found that Tregs in CRC TME comprise mainly of $FoxP3^+Helios^+$ Tregs, which were significantly higher in the TME compared with normal tissue and periphery (5.7 ± 0.6 vs. 6.6 ± 0.5 vs. 20.5 ± 2.3, **Figure 1E**).

$FoxP3^{high}$ Tregs have been previously identified as suppression-competent, while $FoxP3^{low}$ T cells identified as non-suppressive Tregs (14). Therefore, we investigated

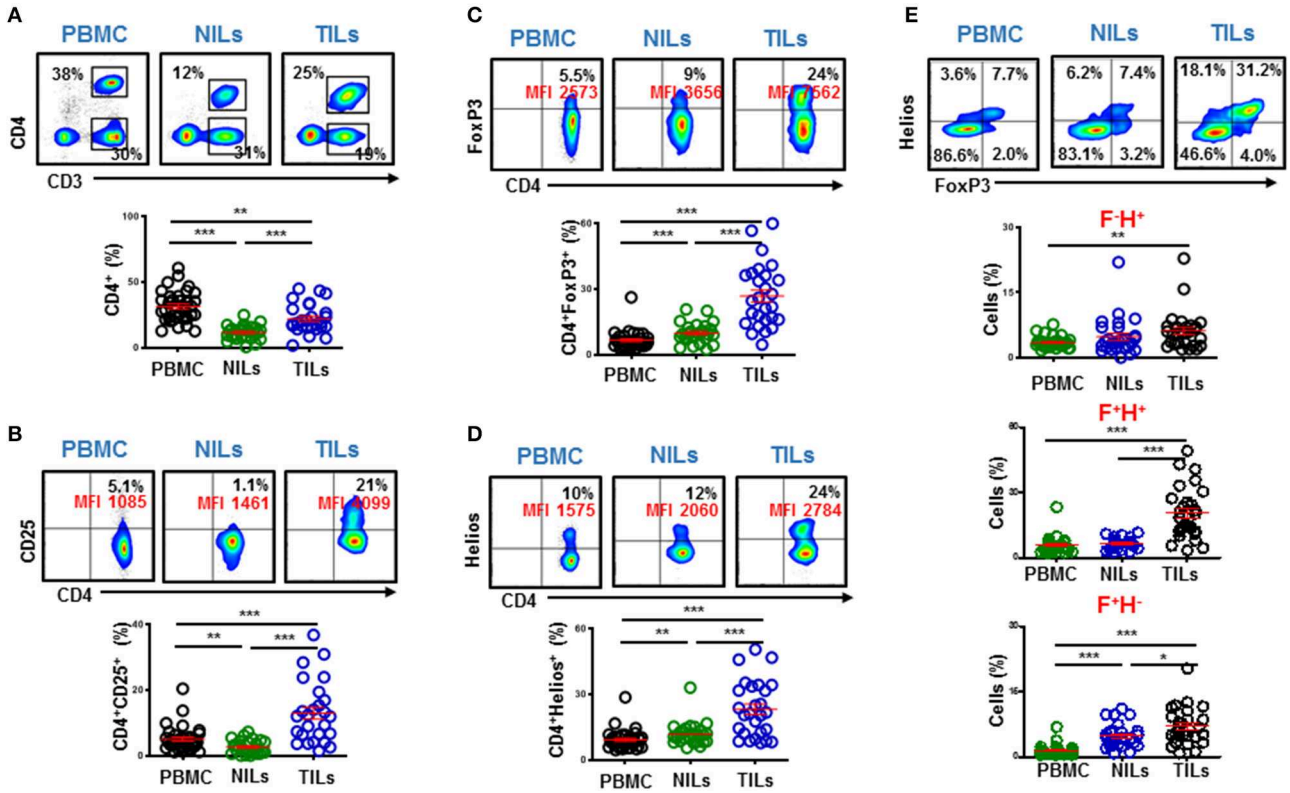

FIGURE 1 | Levels of circulating and tumor-infiltrating CD4$^+$ T cells in colorectal cancer patients and expression levels of T regulatory cell-related markers. PBMC from 34 colorectal cancer (CRC) patients and immune cells isolated from colorectal tumor tissues (TILs) and paired, adjacent non-tumor normal colon tissues (NILs) from 27 patients were stained for CD4$^+$ T cell markers and Treg-related markers including CD25, FoxP3, and Helios. Representative flow cytometric plots and scatter plots show the levels of CD3$^+$CD4$^+$ T cells in PBMC, NILs, and TILs from CRC patients **(A)**. Representative flow cytometric and scatter plots show expression levels of CD25 **(B)**, FoxP3 **(C)**, and Helios **(D)** in CD4$^+$ T cells in PBMC, NILs, and TILs. Representative flow cytometric plots show FoxP3 and Helios co-expression and scatter plots show differences in levels of CD4$^+$FoxP3$^{-/+}$Helios$^{-/+}$ subsets in PBMC, NILs and TILs **(E)**. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

Helios expression within FoxP3^{high} and FoxP3^{low} populations to ascertain the potential suppressive characteristics of the FoxP3$^+$Helios$^+$ subpopulation, accumulated in CRC tumors. We found that CD4$^+$FoxP3^{high} Tregs express significantly higher levels of Helios than CD4$^+$FoxP3^{low} cells in PBMC, NILs, and TILs (PBMC; 58.8 ± 2.6 vs. 88.1 ± 1.7, NILs; 41.4 ± 3.7 vs. 65.1 ± 3.9 & TILs; 58.0 ± 3.5 vs. 80.9 ± 2.3, **Figure 2**). Next, we investigated differences in expression levels of different inhibitory ICs between FoxP3^{low} and FoxP3^{high} Tregs. Interestingly, we found that CTLA-4 and TIM-3 were also expressed at significantly higher levels on CD4$^+$FoxP3^{high} Tregs than CD4$^+$FoxP3^{low} T cells in PBMC, NILs, and TILs (CTLA-4: PBMC; 15.1 ± 2.7 vs. 36.7 ± 3.6, NILs; 48.5 ± 5.2 vs. 77.6 ± 4.8, TILs; 64.4 ± 4.7 vs. 88.3 ± 1.5 & TIM-3: PBMC; 0.6 ± 0.1 vs. 1.0 ± 0.3, NILs; 11.3 ± 2.5 vs. 22.7 ± 3.6, TILs; 21.6 ± 3.7 vs. 38.2 ± 4.7, **Figure 2**). However, PD-1 did not show any significant differences in expression levels between CD4$^+$FoxP3$^{low/high}$ NILs and TILs, but it was significantly lower on CD4$^+$FoxP3^{high} T cells than CD4$^+$FoxP3^{low} T cells in circulation (PBMC; 27.5 ± 2.5 vs. 19.1 ± 2.4, **Figure 2**).

We also compared the mean fluorescence intensity (MFI) of the different markers between CD4$^+$FoxP3^{low} and CD4$^+$FoxP3^{high} Tregs (**Figure 2C**). We found that the differences in population frequencies were also reflected in differences in MFI. The MFI for Helios was significantly higher in CD4$^+$FoxP3^{high} Tregs than CD4$^+$FoxP3^{low} T cells in PBMC (1928 ± 36.6 vs. 2253 ± 52.1), NILs (2457 ± 108.2 vs. 2656 ± 163.7) and TILs (2374 ± 97.4 vs. 2759 ± 156.9). CTLA-4 and TIM-3 also showed similar patterns (CTLA-4: PBMC; 2384 ± 64.5 vs. 2701 ± 73.2, NILs; 2492 ± 221.1 vs. 5197 ± 365.8, TILs; 2922 ± 188.5 vs. 5797 ± 501.5 and TIM-3: PBMC; 621 ± 19.5 vs. 771 ± 44.5, NILs; 1546 ± 84.9 vs. 1889 ± 145.0, TILs; 1708 ± 69.6 vs. 2203 ± 161.2), while PD-1 did not show any significant differences in MFI between CD4$^+$FoxP3^{low} and CD4$^+$FoxP3^{high} Tregs in PBMC, NILs and TILs (**Figure 2C**).

High Expression of Immune Checkpoints on Intratumoral CD4$^+$ T Cells

Immune checkpoints are expressed on activated or exhausted T cells (16). To find out the functional state of infiltrating T cells in the colorectal TME, we investigated IC expression on different CD4$^+$ T cell subsets. We found that key inhibitory ICs, including PD-1, CTLA-4, TIM-3 and LAG-3 were highly expressed on CD4$^+$ TILs (**Figure 3**). These

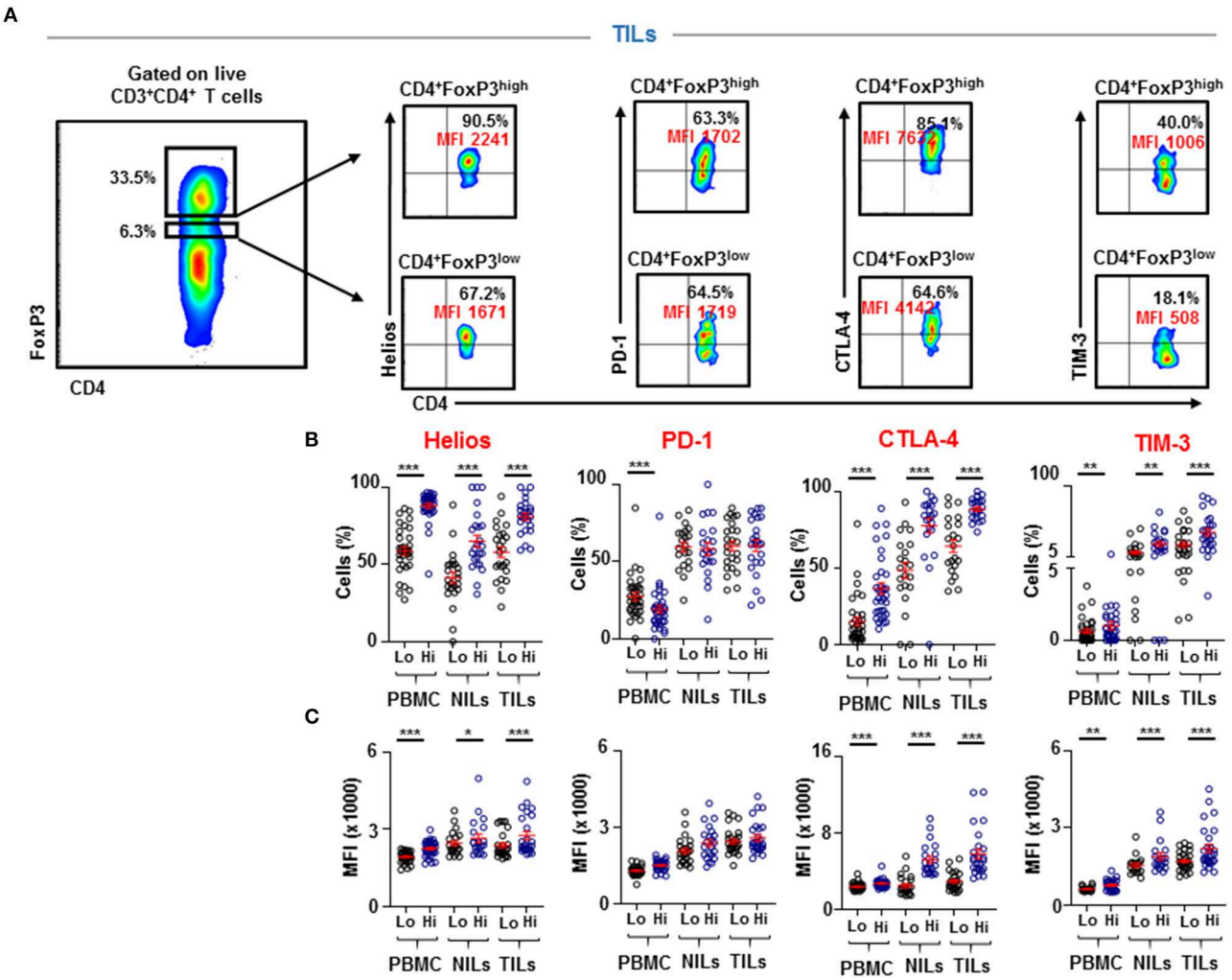

FIGURE 2 | Differences in Helios and immune checkpoint expression on CD4$^+$FoxP3^{low} and CD4$^+$FoxP3^{high} PBMC, NILs, and TILs from CRC patients. CD4$^+$FoxP3^{low} and CD4$^+$FoxP3^{high} Tregs were gated in PBMC, NILs, and TILs to investigate Helios, PD-1, CTLA-4, and TIM-3 expressions. Representative flow cytometric plots show gating strategy for defining CD4$^+$FoxP3$^{low/high}$ Tregs and the expression levels of Helios, PD-1, CTLA-4, and TIM-3, in TILs isolated from CRC patients **(A)**. Scatter plots show differences in expression level **(B)** and Mean Fluorescence Intensity (MFI) **(C)** of Helios, PD-1, CTLA-4, and TIM-3 between CD4$^+$FoxP3^{low} and CD4$^+$FoxP3^{high} PBMC, NILs, and TILs from CRC patients. $^{***}P < 0.001$, $^{**}P < 0.01$.

ICs were expressed at significantly lower levels in periphery compared to normal colon tissue and showed elevated expression levels in the TME (PD-1: PBMC; 14.9 ± 1.1 vs. NILs; 48.1 ± 4.8 vs. TILs; 57.8 ± 5.7, CTLA-4: 4.8 ± 0.8 vs. 22.7 ± 2.4 vs. 45.9 ± 5.7, TIM-3: 0.5 ± 0.1 vs. 7.5 ± 1.0 vs. 23.2 ± 3.2 & LAG-3: 0.4 ± 0.1 vs. 1.8 ± 0.4 vs. 2.7 ± 0.5, **Figure 3**). Moreover, it is noteworthy that the overall expression levels of PD-1 were highest in tissues and in periphery, followed by CTLA-4 and TIM-3, while LAG-3 showed lowest overall expression on CD4$^+$ T cells compared to other ICs (**Figure 3**).

We also investigated co-expression of PD-1 with other ICs in PBMC, NILs, and TILs. We found that PD-1 was mainly co-expressed with CTLA-4 and TIM-3 in CD4$^+$ TILs (**Figures 4A,B**). In contrast, although CD4$^+$PD-1$^+$LAG-3$^+$ T cells were significantly higher in NILs and in TILs compared to PBMC, the majority of CD4$^+$PD-1$^+$ T cells do not co-express LAG-3 (**Figure 4C**).

CTLA-4, TIM-3, and LAG-3 Are Mainly Expressed on FoxP3$^+$Helios$^+$ Tregs in the Tumor Microenvironment

A plausible approach to evoke potent antitumor immune responses without triggering autoimmunity is to target terminally differentiated Tregs (24). Previous studies have reported overexpression of various ICs on Tregs including constitutive expression of CTLA-4 (25–27). We wanted to

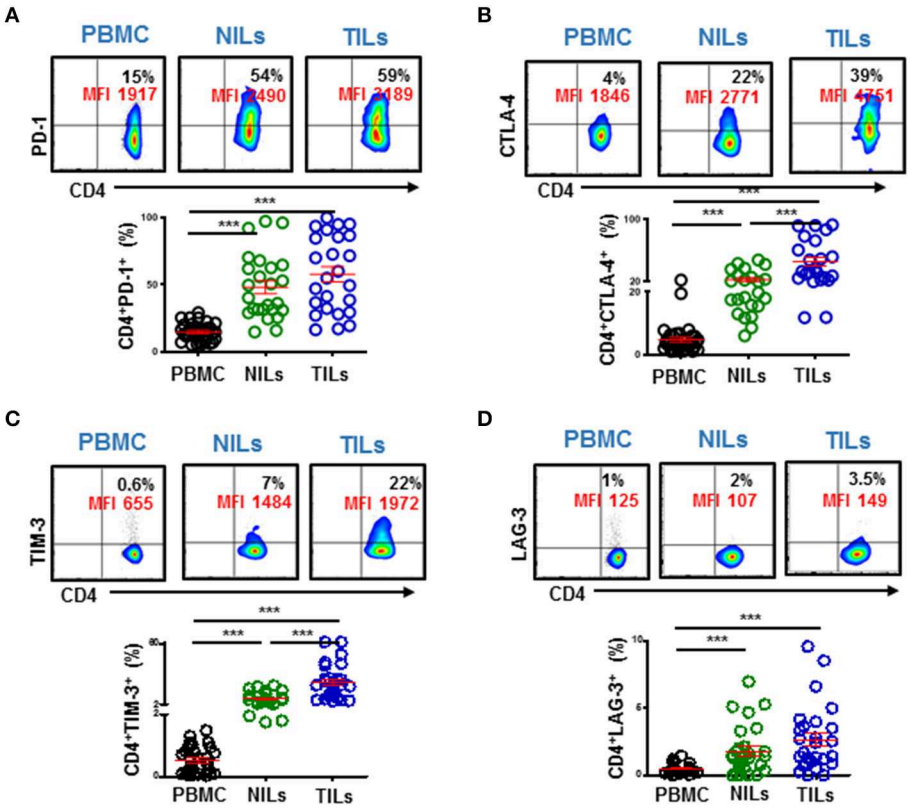

FIGURE 3 | Immune checkpoint expression on CD4+ T cells in PBMC, NILs, and TILs from CRC patients. PBMC, NILs, and TILs were stained for CD3, CD4, and key immune checkpoints. Representative flow cytometric plots and scatter plots show differences in expression levels of PD-1 **(A)**, CTLA-4 **(B)**, TIM-3 **(C)**, and LAG-3 **(D)** on CD4+ T cells in PBMC, NILs, and TILs. ***$P < 0.001$.

identify which Treg subpopulations upregulate inhibitory ICs in the colorectal TME based on FoxP3 and Helios expression. We found that key inhibitory ICs are differentially upregulated on various intratumoral Treg subsets, compared to $CD4^+FoxP3^-Helios^-$ non-Tregs (**Figures 5A–D**). In TILs, PD-1 was mainly expressed on $CD4^+FoxP3^-Helios^+$ (60.3 ± 4.5) and $CD4^+FoxP3^+Helios^-$ Tregs (59.7 ± 5.5), compared to $CD4^+FoxP3^+Helios^+$ (49.5 ± 6.1) and $CD4^+FoxP3^-Helios^-$ (50.6 ± 5.5) (**Figure 5A**). CTLA-4 and LAG-3 were mainly expressed on both $CD4^+FoxP3^+Helios^+$ (CTLA-4; 78.5 ± 3.8 & LAG-3; 6.8 ± 0.9) and $CD4^+FoxP3^+Helios^-$ Tregs (CTLA-4; 72.8 ± 4.1 & LAG-3; 6.2 ± 0.8), compared to $CD4^+FoxP3^-Helios^+$ TILs (CTLA-4; 48.7 ± 5.6 & LAG-3; 4.4 ± 0.9) and $CD4^+FoxP3^-Helios^-$ TILs (CTLA-4; 21.8 ± 3.5 & LAG-3; 0.5 ± 0.1) (**Figures 5B,D**). While, TIM-3 was mainly expressed on $CD4^+FoxP3^+Helios^+$ TILs (44.5 ± 4.6), compared to $CD4^+FoxP3^-Helios^+$ (22.8 ± 3.4), $CD4^+FoxP3^+Helios^-$ TILs (25.9 ± 3.4) and $CD4^+FoxP3^-Helios^-$ TILs (5.9 ± 1.8) (**Figure 5C**).

Notably, inhibitory ICs showed different expression patterns on $CD4^+FoxP3^{-/+}Helios^{-/+}$ in periphery. PD-1 was mainly expressed on $CD4^+FoxP3^+Helios^-$ Tregs in circulation (19.5 ± 2.0), followed by $CD4^+FoxP3^-Helios^+$ T cells (14.0 ± 1.1) and $CD4^+FoxP3^-Helios^-$ T cells (11.3 ± 1.0), while $CD4^+FoxP3^+Helios^+$ Tregs showed lowest PD-1 expression (7.4 ± 0.8) (**Figure 5E**). CTLA-4 was mainly expressed on $CD4^+FoxP3^+Helios^-$ (33.8 ± 3.5) and $CD4^+FoxP3^+Helios^+$ Tregs (30.0 ± 2.7), compared to $CD4^+FoxP3^-Helios^+$ (10.7 ± 1.6) and $CD4^+FoxP3^-Helios^-$ T cells (1.9 ± 0.3). TIM-3 was mainly expressed on FoxP3 and/or Helios-expressing T cells, compared to $CD4^+FoxP3^-Helios^-$ T cells (0.2 ± 0.1) in circulation (**Figure 5E**). In addition, no significant differences were detected in TIM-3 expression on $CD4^+FoxP3^-Helios^+$ (1.1 ± 0.2), $CD4^+FoxP3^+Helios^+$ (1.4 ± 0.4) and $CD4^+FoxP3^+Helios^-$ (1.6 ± 0.4) T cells in circulation (**Figure 5E**). In normal colon tissues, PD-1 expression did not show any significant differences in $CD4^+FoxP3^-Helios^+$ (36.4 ± 5.0), $CD4^+FoxP3^+Helios^+$ (30.2 ± 5.0), $CD4^+FoxP3^+Helios^-$ (33.8 ± 6.4) and $CD4^+FoxP3^-Helios^-$ (33.3 ± 3.8) NILs (**Figure 5F**). CTLA-4 was also mainly expressed on $CD4^+FoxP3^+Helios^-$ (43.9 ± 7.0) and $CD4^+FoxP3^+Helios^+$ NILs (37.9 ± 6.1), compared to $CD4^+FoxP3^-Helios^+$ (20.5 ± 4.8) and $CD4^+FoxP3^-Helios^-$ NILs (11.1 ± 1.9). In contrast, TIM-3 was mainly expressed on $CD4^+FoxP3^+Helios^+$ NILs (16.6 ± 2.9), followed by $CD4^+FoxP3^+Helios^-$ (8.7 ± 2.1) and $CD4^+FoxP3^-Helios^+$

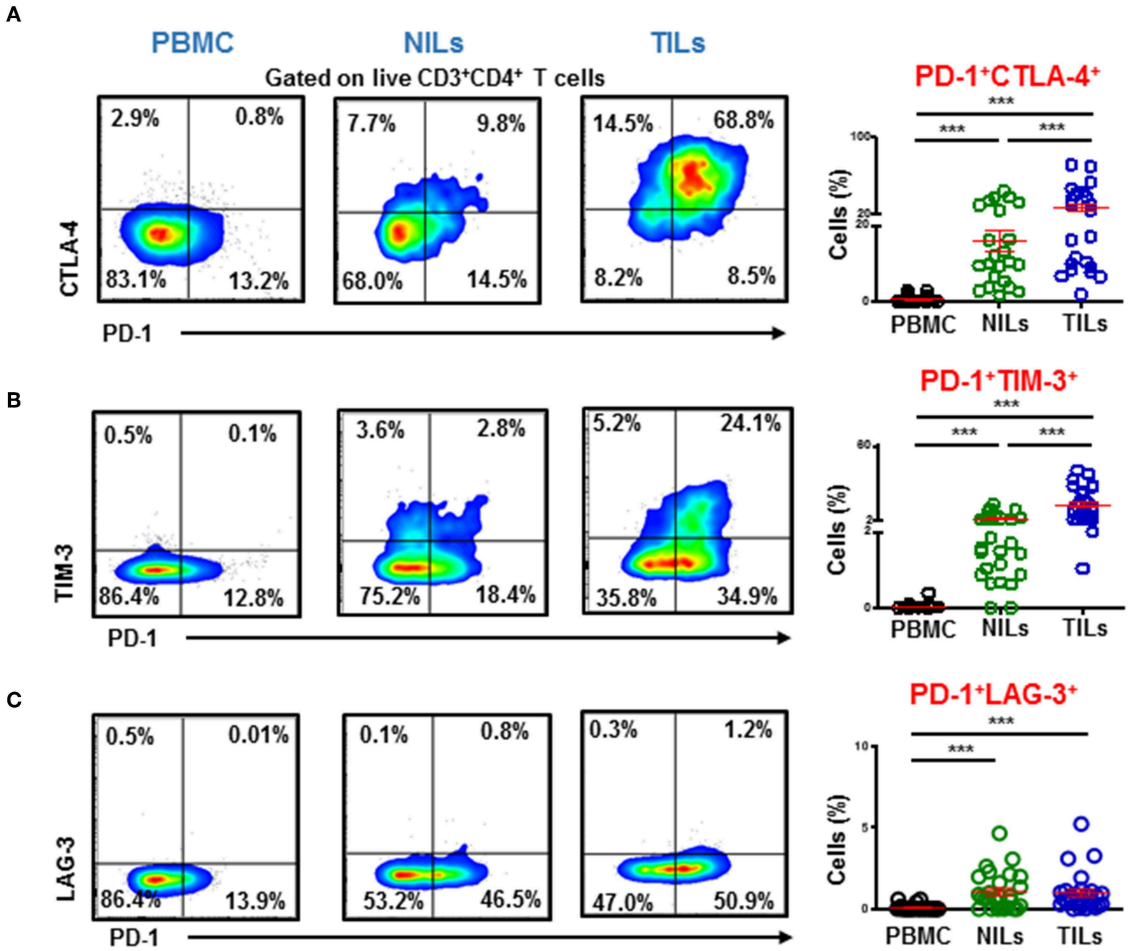

FIGURE 4 | Immune checkpoints co-expression on CD4+ T cells. PBMC, NILs, and TILs isolated from CRC patients were stained for CD3, CD4, and immune checkpoints. Representative flow cytometric and scatter plots show differences in co-expression levels of PD-1$^{-/+}$CTLA-4$^{-/+}$ **(A)**, PD-1$^{-/+}$TIM-3$^{-/+}$ **(B)**, and PD-1$^{-/+}$LAG-3$^{-/+}$ **(C)** on CD4+ T cells in PBMC, NILs, and TILs. ***$P < 0.001$.

(2.1 ± 0.7) NILs, while CD4+FoxP3−Helios− NILs showed minimal TIM-3 expression (1.0 ± 0.2) (**Figure 5F**). Of note, there were no conclusive results to identify the CD4+FoxP3+/−Helios+/− subset with highest LAG-3 expression due to weak overall LAG-3 expression in periphery (data not shown).

Visualization of overall CD4+ T cell infiltrates in CRC tumors and periphery is depicted in **Figure 6**. We generated t-Distributed Stochastic Neighbor Embedding (tSNE) plots from various Treg-related markers and inhibitory ICs in PBMC, NILs, and TILs from CRC patients. We confirmed that FoxP3, Helios, PD-1, TIM-3, and CTLA-4 expression levels are increased in CD4+ TILs, while CD4+ T cells showed lower Treg-related markers and IC expression in PBMC and NILs (**Figure 6A**). In addition, CD4+ TILs co-expressed multiple ICs compared to PBMC and NILs (**Figure 6B**). Helios, PD-1 and TIM-3 were also expressed on CD4− T cells and showed elevated expression on CD4− TILs (**Figure 6B**).

Patients With Advanced Stage Disease Have Higher Levels of CD4+CTLA-4+ T Cells in Circulation

We compared the levels of CD4+ T cell subsets in circulation, NILs and TILs between CRC patients presenting with early and advanced pathologic stages (**Figures 7A–C**). We combined patients with stages I and II (PBMC; $n = 15$, NILs/TILs; $n = 11$) and compared with those with stages III and IV advanced stage (PBMC; $n = 19$, NILs/TILs; $n = 16$). We found that the levels of circulating CD4+ T cells were similar between patients with early or advanced stages. Intestinally, there was a significant increase in levels of CD4+CTL-4+ T cells only in circulation of patients with advanced stage (4.0 ± 1.3 vs. 5.4 ± 1.1, **Figure 7A**). The constitutive expression of CTLA-4 on CD4+ Tregs suggests correlation of elevated levels of CTLA-4+ Tregs with CRC disease progression. However, other T cell subsets, including CD4+FoxP3+ Tregs,

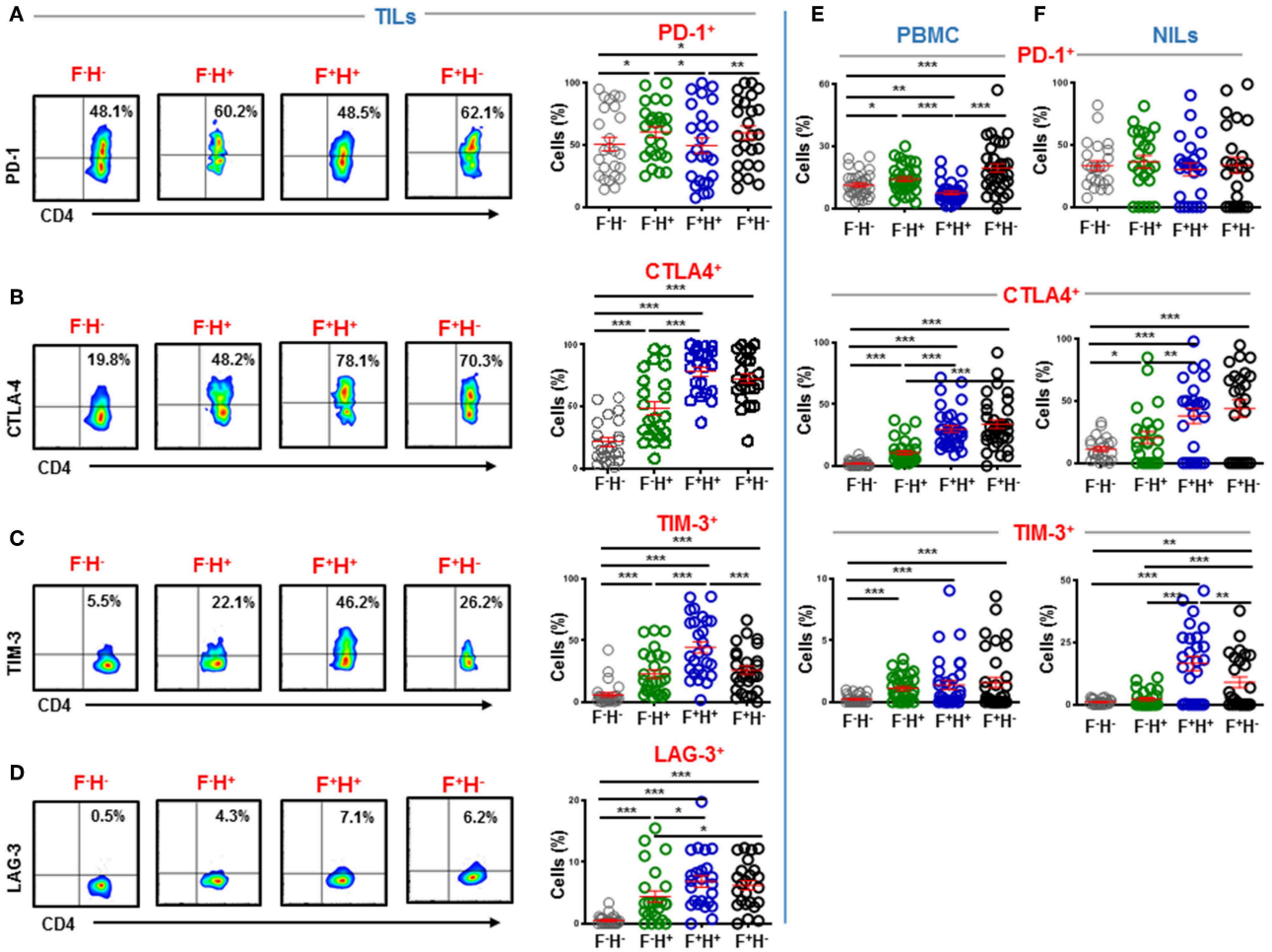

FIGURE 5 | Immune checkpoint expression on FoxP3 and Helios subsets in CD4+ TILs, PBMC, and NILs. TILs isolated from CRC patients were stained for CD3, CD4, immune checkpoints and FoxP3 and Helios. Representative flow cytometric plots and scatter plots show differences in expression levels of PD-1 **(A)**, CTLA-4 **(B)**, TIM-3 **(C)**, and LAG-3 **(D)** in CD4+FoxP3−/+Helios−/+ TILs subsets. Scatter plots show differences in expression levels of PD-1, CTLA-4, and TIM-3 in CD4+FoxP3−/+Helios−/+ PBMC **(E)** and NILs **(F)** from CRC patients. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

did not show any correlation with disease progression (data not shown).

Levels of CD4+ T Cells Are Similar in Patients Exhibiting Varying Tumor Budding Status

Tumor budding in CRC has been associated with poor disease outcomes in various studies (28). We divided our cohort into three groups based on tumor budding data; low (PBMC; $n = 13$, NILs/TILs; $n = 11$), intermediate (PBMC; $n = 11$, NILs/TILs; $n = 7$) and high (PBMC; $n = 10$, NILs/TILs; $n = 9$), and compared levels of various T cell subsets in circulation, NILs and TILs between them. We did not find any significant differences between CD4+ T cell subsets in circulation, NILs or TILs (**Figures 7D–F**). Moreover, there were no significant differences in the levels of other CD4+ T cell subsets, including TIM-3-expressing CD4+ T cells, between patients with different tumor budding status (data not shown).

DISCUSSION

Studies have reported correlations between increased T cell infiltration of various tumors and improved responses to therapies and favorable disease outcomes (29). Interferon-γ-secreting cytotoxic T cells, T-helper 1 (Th1) cells, natural killer cells and macrophages polarized to an M1 phenotype and DC1 dendritic cells are largely associated with favorable anti-tumor immune responses (30–32). While, Th2 cells, M2 macrophages, DC2 dendritic cells, myeloid derived suppressor cells (MDSC) and IL-10 and TGFβ releasing FoxP3+ Tregs are associated with immunosuppression (33, 34).

In CRC patients, high infiltration of CD3+ T cells, Th1 cells and cytotoxic T cells in the tumor center and invasive margins correlated with improved overall survivals and disease-free survivals, while lower T cell density was associated with poor prognosis (35, 36). In addition, high CD8+ tumor-infiltrating T cells were shown to be a favorable prognostic factor for right-sided colon tumors (37) and increased levels of CD4+

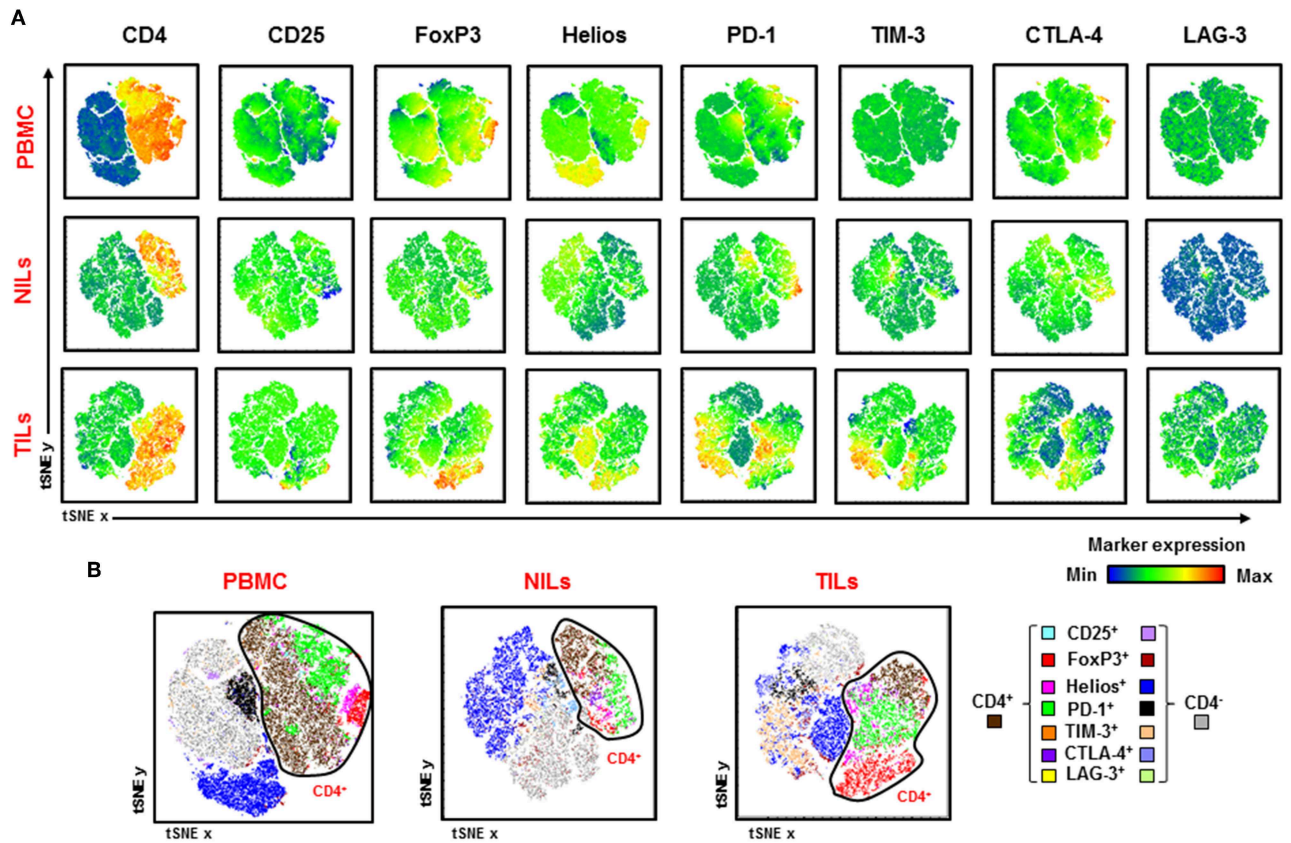

FIGURE 6 | Visualizing expression of Treg-related markers and immune checkpoint expression in PBMC, NILs, and TILs from CRC patients. Flow cytometric data from PBMC, NILs, and TILs were merged to create single t-Distributed Stochastic Neighbor Embedding (tSNE) maps. Cells (dots) are plotted according to expression of CD4, CD25, FoxP3, Helios, PD-1, TIM-3, CTLA-4, and LAG-3 in PBMC, NILs, and TILs from CRC patients **(A)**. tSNE maps show combined co-expression of the different Treg-related markers and immune checkpoints on $CD4^+$ and $CD4^-$ T cells in PBMC, NILs and TILs from CRC patients **(B)**.

and $CD8^+$ T cells in colorectal TME were shown to correlate with improved response to chemo-radiotherapy (38). Moreover, the presence of effector memory T cells within CRC tumors, defined by the presence of CD3, CD8, CD45RO, CCR7, CD28, and CD27 expression, was associated with absence of signs of early metastatic invasion (39). Therefore, evidence of an active immune response in the CRC TME was shown to be associated with prolonged survival (39).

We found that $CD4^+$ T cells were significantly higher in colorectal tumors, compared with normal colon tissues. $CD4^+$ T cells in circulation comprise mainly of naïve T cells, while in tissues comprise mainly of memory T cells. Galon et al. proposed that the immune landscape of CRC tumors can be considered as a robust predictor of patient survival and it may be used for histopahlogical classification of CRC tumors; they found that patients with high immune cell densities within the tumor and at invasive margins did not show recurrence (40). Importantly, transcriptomic profiling of immune subsets found in CRC tumors confirmed that immune cell infiltrates can affect disease outcomes as patients with prolonged disease-free survivals had distinct expression of genes related to cytotoxic T cells, T helper molecules and chemokine-related genes than patients with adverse disease outcomes (35). Therefore, comprehensive investigations are required to ascertain the role of immune cells in the TME and their effects on clinical outcomes of CRC patients. We reported high levels of different ICs and Treg-related markers in CRC TME, which would suggest their potential roles in carcinogenesis. Mechanisms of expansion/proliferation or trafficking of these $CD4^+$ T cell subsets into tumor sites warrants further investigations.

Pre-existing Tregs in the TME expand upon antigen-specific activation in the presence of TGF-β and IL-10, which are found at high levels within the TME (41, 42). We found that $CD4^+CD25^+FoxP3^+$ Tregs accumulate in colorectal tumors at significantly higher levels, compared to periphery and adjacent colon normal tissues. Moreover, these Tregs expressed high levels of Helios, indicative of highly suppressive and stable Treg function (43). Studies have shown that $FoxP3^+Helios^+$ Tregs have enhanced immunosuppressive characteristics, compared with $FoxP3^+Helios^-$ Tregs (44–46).

Majority of studies have associated Tregs with poor clinical outcomes in different cancers including CRC (47, 48), however some studies have also associated these with better prognosis in CRC patients (49–51). Saito et al. proposed that these results

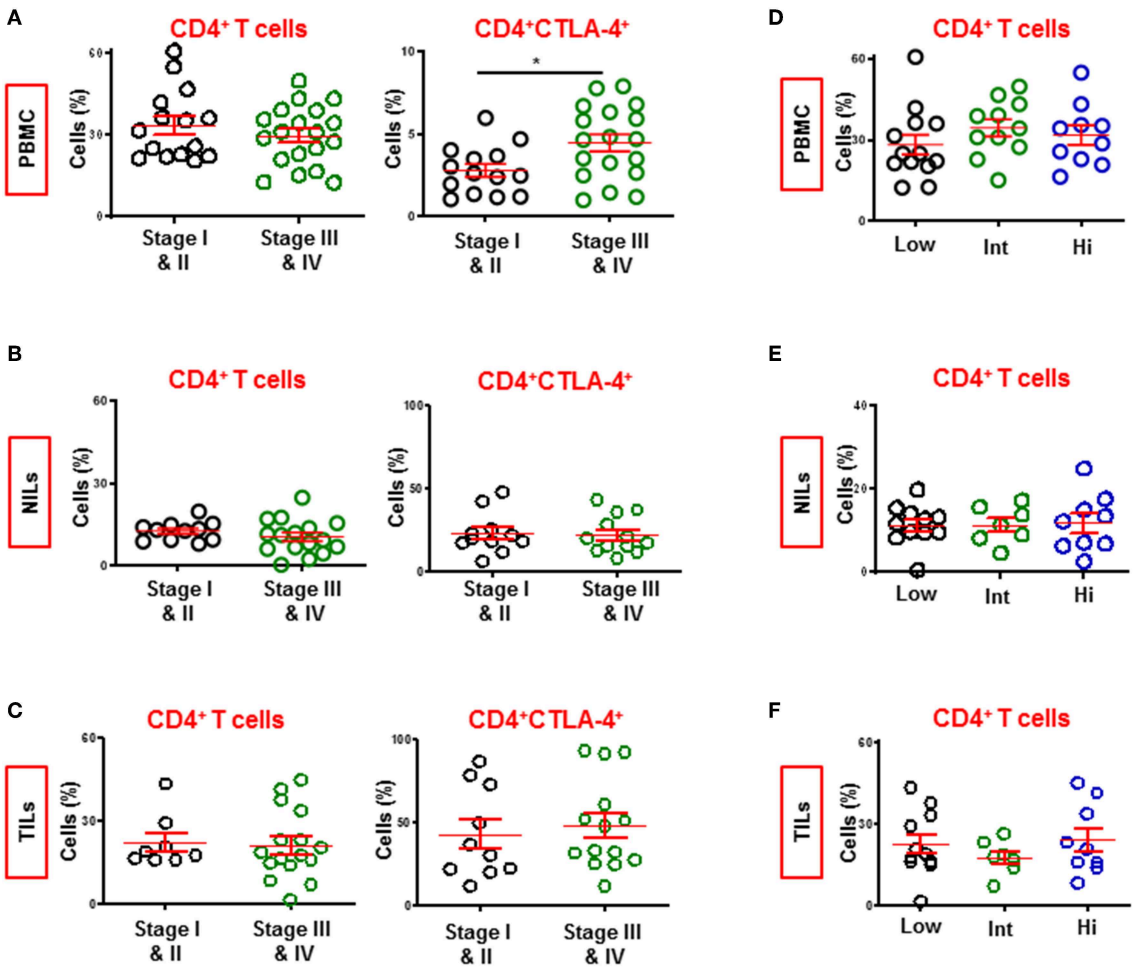

FIGURE 7 | Levels of $CD4^+$ and $CD4^+CTLA\text{-}4^+$ T cells in CRC patients with different staging and tumor budding. Patients were divided into two groups based on pathologic staging; early stage (Stage I and II) and advanced stage (Stage III and IV). Scatter plots show differences in levels of $CD4^+$ and $CD4^+CTLA\text{-}4^+$ T cells in PBMC **(A)**, NILs **(B)**, and TILs **(C)** between CRC patients with early and advanced stages. Patients were also divided into three groups based on degree of tumor budding; low, intermediate and high. Scatter plots show differences in levels of $CD4^+$ T cells in PBMC **(D)**, NILs **(E)**, and TILs **(F)** between CRC patients. $^*P < 0.05$.

could be attributed to different subsets of tumor-infiltrating $FoxP3^+$ Tregs, which include $FoxP3^{high}$ and $FoxP3^{low}$ Tregs; the former representing stable FoxP3 expression, while $FoxP3^{low}$ non-suppressive Tregs secrete inflammatory cytokines and henceforth may be associated with improved clinical outcomes in CRC (14). In addition, it has been shown that tumor-infiltrating $FoxP3^{high}CD45RA^-$ effector Tregs, which express PD-1, are highly activated, express high levels of CTLA-4 and are associated with hyper progressive disease in patients with advanced gastric cancer (52). Based on these findings, we investigated differences in Helios and other IC expression between $FoxP3^{low}$ and $FoxP3^{high}$ PBMC, NILs, and TILs. We found that $FoxP3^{high}$ Tregs express Helios at significantly higher levels than $FoxP3^{low}$ T cells in the TME and in periphery of CRC patients, strengthening that Helios is a vital marker for suppressive Tregs in CRC. Additionally, $FoxP3^{high}$ Tregs also showed significantly higher IC expression in the TME, indicating their highly activated states.

ICs are expressed on activated T cells including both Teff cells and Tregs (10), they are also highly expressed on dysfunctional and exhausted T cells, characterized by defective effector function and proliferation (53, 54). IC expression on T cells represent early activation or exhaustion due to prolonged exposure to residing antigens, which ultimately attenuate their effector functionality (55). Studies have shown that multiple inhibitory receptors are associated with T cell exhaustion but the specific mechanisms that administer their transcriptional and epigenetic development or their phenotypic identification remain to be fully elucidated. Notably, recent studies have proposed the HMG-box transcription factor TOX as a critical regulator of T cell exhaustion, which may be used to identify exhausted T cells (56). Additionally, investigating expression of other key transcription factors associated with T cell exhaustion, such as NFAT, EOMES, T-bet, FOXO1, and FOXP1 (55) in different IC-expressing $CD4^+$ T cells would also ascertain their exhaustive states.

CTLA-4 is constitutively expressed on Tregs and modulates their functionality (57). Additionally, Tregs in the TME upregulate other multiple ICs including PD-1, LAG-3, TIM-3, and TIGIT (58); FoxP3$^+$ Tregs expressing TIM-3/LAG-3 and PD-1 were shown to be highly suppressive (25, 59). TIM-3 expression on Tregs has been previously reported to show higher ability to suppress Th17 cells than TIM-3$^-$ Tregs, while both can effectively suppress Th1 proliferation (60). We found that in the CRC TME, TIM-3 is mainly expressed on FoxP3$^+$Helios$^+$ Tregs, hence more potent suppressors of Th17 responses. In addition, we showed that PD-1, TIM-3, CTLA-4, and LAG-3 were mainly expressed on CD4$^+$FoxP3$^+$ Tregs in the TME, further reinforcing the suppressive role of these cells in the TME. tSNE representation, which enables visualization of high-dimensional data on a single bivariate plot, also confirmed the accumulation of Tregs and elevated IC expression on CD4$^+$ T cells in the TME. Moreover, high IC expression corresponded with Treg populations in TILs.

Tumor budding is associated with vascular invasion and prognosis in colorectal cancer (61, 62). This study cohort consisted of CRC displaying different tumor budding characteristics, ranging from low to high. However, we did not find any differences in the levels of CD4$^+$ T cells in periphery or TME across all patients with varying tumor budding status; thereby suggesting CD4$^+$ T cells are not associated with tumor budding in CRC.

Following the accomplishments of PD-1 and CTLA-4 blockade, TIM-3 and LAG-3 are currently being explored in various pre-clinical and clinical trials to promote effective anti-tumor immunity for clinical benefits (63). This study provides comprehensive and simultaneous comparisons of expression levels of different ICs on CD4$^+$ T cells, including Tregs, in the TME and periphery of CRC patients. IC inhibitors significantly improved survival in patients with MSI-H metastatic CRC. However, a significant proportion of patients show minimal response and do not benefit from ICIs (64). Additionally, the percentage of CRC patients who exhibit MSI-H/dMMR is generally low, around 12–15% of all cases (65), like in this study cohort (**Table 1**). Equating immune profiles of these patients with those who do not show microsatellite instability to find differences would therefore require a much larger patient pool. Moreover, the long-term prognosis of patients with advanced stage CRC remains poor despite efforts to develop novel chemotherapeutic and targeted therapy regimens. Predictive biomarkers for successful IC inhibition with clinical benefits are a necessity in such instances. Expression of ICs and their ligands in the TME have been proposed as such predictive biomarkers (66), and better understanding of the immune components can therefore assist in identifying robust biomarkers for response to therapy and also assist in targeted-therapies, tailor made on individual patient basis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Qatar Biomedical Research Institute, Doha, Qatar (Protocol no. 2018-018) and Hamad Medical Corporation, Doha, Qatar (Protocol no. MRC-02-18-012). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ST performed experimental work, data analysis, and wrote the manuscript. KM assisted with data acquisition and analysis. KM, MA-D, and MK contributed to sample collection, acquisition of patients' clinical data, and revising the manuscript. MA assisted in designing the study, contributed to sample collection, and revised the manuscript. EE conceived the idea, designed the study, obtained fund, analyzed and interpreted data, and wrote and revised the manuscript. All authors were involved in the final approval of the manuscript.

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REVIEW
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One, No One, and One Hundred Thousand: T Regulatory Cells' Multiple Identities in Neuroimmunity

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As the Nobel laureate Luigi Pirandello wrote in his novels, identities can be evanescent. Although a quarter of a century has passed since regulatory T cells (Treg) were first described, new studies continue to reveal surprising and contradictory features of this lymphocyte subset. Treg cells are the core of the immunological workforce engaged in the restraint of autoimmune or inflammatory reactions, and their characterization has revealed substantial heterogeneity and complexity in the phenotype and gene expression profiles, proving them to be a most versatile and adaptive cell type, as exemplified by their plasticity in fine-tuning immune responses. Defects in Treg function are associated with several autoimmune diseases, including multiple sclerosis, which is caused by an inappropriate immune reaction toward brain components; conversely, the beneficial effects of immunomodulating therapies on disease progression have been shown to partly act upon the biology of these cells. Both in animals and in humans the pool of circulating Treg cells is a mixture of natural (nTregs) and peripherally-induced Treg (pTregs). Particularly in humans, circulating Treg cells can be phenotypically subdivided into different subpopulations, which so far are not well-characterized, particularly in the context of autoimmunity. Recently, Treg cells have been rediscovered as mediators of tissue healing, and have also shown to be involved in organ homeostasis. Moreover, stability of the Treg lineage has recently been addressed by several conflicting reports, and immune-suppressive abilities of these cells have been shown to be dynamically regulated, particularly in inflammatory conditions, adding further levels of complexity to the study of this cell subset. Finally, Treg cells exert their suppressive function through different mechanisms, some of which—such as their ectoenzymatic activity—are particularly relevant in CNS autoimmunity. Here, we will review the phenotypically and functionally discernible Treg cell subpopulations in health and in multiple sclerosis, touching also upon the effects on this cell type of immunomodulatory drugs used for the treatment of this disease.

Keywords: neuroimmunity, multiple sclerosis, Treg-regulatory T cell, FoxP3, Treg heterogeneity, immune regulation

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INTRODUCTION

The immune system is called to respond to the environment at each beat of the heart, at each breath, and at each breaching of the organism's surface. In a healthy organism, should an exogenous and potentially dangerous entity gain access, the immediate response of the immune system's defense mechanisms is activated, and clearance of the pathogen is achieved, millions of times a day (1). In addition, immune cells interact continuously with the trillions of bacteria, fungi, and viruses which inhabit, peacefully, the intestines. The interactions with the intestinal microbiota are the second process, after thymic selection, that shapes the immune system. This notion has become clearer in recent years with the knowledge that dysbiosis, an alteration of the physiological composition of the microbial community, underlies the generation of an inflammatory environment which may raise the level of basal immune cell activation thus predisposing to the initiation of inappropriate and uncontrolled immune responses, leading to chronic inflammation and disease (2).

Normally, immune reactions are kept in check by multiple mechanisms which control the magnitude of the response, so escalation to overt inflammation and tissue damage are avoided. However, additional stimuli which may activate immune T cells come also from within, since all the organism's tissues are also highly immunogenic, as exemplified by the immediate rejection of transplants between incompatible individuals. Conveniently, efficient mechanisms of selection during T cell development in the thymus are in place for purging autoreactive cells from the repertoire, and clonal deletion eliminates most of them (3, 4). Inevitably, though, some autoreactive T cells escape this negative selection and circulate in the periphery, and indeed they have been shown to be present at similar frequencies both in healthy individuals and in those afflicted by autoimmune diseases (5–7). But autoimmunity leading to pathology represents the exception rather than the rule, so clearly these autoreactive cells are kept in check in the periphery. Several immune mechanisms exist which mediate restraint of autoreactivity (8), and among these are FoxP3$^+$ T regulatory (Treg) cells, whose features and roles in the aberrant immune response which underlies multiple sclerosis (MS) will be discussed.

TREG CELLS IN HEALTH

The FoxP3 Transcription Factor

Treg cells were initially described as a CD4$^+$CD25^{high} T cell population whose depletion is associated with the development of severe multi-organ autoimmunity, and were defined by the expression of the transcription factor forkhead box P3 (FoxP3) (9–11).

FoxP3 is capable of binding the astounding number of 2,800 genomic sites, and interacts with more than 360 factors to form a large protein complex (12–15). Four evolutionary conserved intronic cis-elements in the FoxP3 locus regulate the *foxp3* gene expression: conserved non-coding sequence (CNS) 3 is indispensable for the initiation of FoxP3 transcription through the recruitment of c-Rel; CNS2 enables the stable expression of FoxP3 in actively proliferating Tregs, and CNS1 is key for the extrathymic induction of Tregs in the periphery, and contains binding sites for TGF-β (16). Last to be discovered, but actually the pioneering element, is CNS0, crucial for the establishment of the earliest epigenetic modification controlling FoxP3 expression (17). Interestingly, methylation at these crucial sites is affected by cytokine signaling and by environmental cues, thus it is possible that the inflammation which accompanies autoimmunity may have an impact on this "basic" epigenetic regulation and stability of FoxP3 (18).

Stable FoxP3 expression also relies on epigenetic modifications of the Treg-specific demethylated region (TDSR), a non-coding region in the first intron of the *foxP3* gene locus (19, 20), and this has become the marker of "true" Treg cells, allowing discrimination from activated CD4$^+$CD25$^+$FoxP3$^+$ cells. The presence of DNA hypomethylation at Treg signature genes contributes to the maintenance of lineage stability, and does not occur in activated cells which transiently express FoxP3 and which lack suppressive abilities (21).

Additionally, similar to most transcription factors, FoxP3's function can be modulated by post-translational modifications (such as ubiquitination, acetylation, and phosphorylation), which couple extracellular cues to adjustments of transcriptional programmes [for a review see (22, 23)].

In humans, several splicing variants of FoxP3 have been described (24). The splicing variant containing exon 2 (FoxP3-E2) is the better equipped for interaction with RORα and RORγt, two transcription factors involved in Th17 specification (25, 26). Metabolic and cytokinic factors determine alternative splicing, and we and others have shown that, in patients with MS, Treg cells express reduced levels of FoxP3-E2 and are thus deprived of an auxiliary level of regulation (27, 28).

The Treg phenotype needs to be "locked in" and stabilized, since these cells are self-reactive and their conversion into conventional effector cells would unleash a dangerous army of autoimmune effectors (29). So how do Treg cells resist acquisition of conventional T (Tconv) cell properties, in inflammatory environments? FoxP3 prevents the expression of genes encoding effector cytokines by acting as a repressor or an activator and through the physical interaction with other transcription factors (30, 31). These aspects are discussed below.

TREG CELL DEVELOPMENT

In vivo, most Treg cells develop in the thymus (tTreg) during positive selection as an alternative to conventional CD4$^+$ T cells, but they also may be generated in the periphery (pTreg) through extra-thymic conversion of conventional T cells.

In the thymus, T cells recognizing self-antigens are deleted, except for a small fraction of cells, which is pushed down the path of Treg differentiation, largely due to the avidity of the MHC-peptide interaction that their TCR establishes with cortical thymocytes (32). Specifically, diversion in the Treg lineage occurs when the affinity of the TCR for self-peptides ligands falls between the range of positive selection of conventional

T cells and negative selection of T cells carrying TCRs with high affinity. Thus, strong TCR signaling is the prerequisite for Treg development (33). In addition to TCR signaling, other factors, such as signaling through CD28 (34), the presence of IL-2 (35) and of TGF-β (36) are required for Treg differentiation. Following this first step of TCR-dependent maturation, CD25 upregulation occurs, making tTreg precursors responsive to the IL-2 signals, which are transduced by STAT5 and are necessary for the induction of the master regulator FoxP3 (37). Interestingly, in the thymus there is limited availability of IL-2, which may be further reduced following sequestration by mature activated Treg cells recirculating to the thymus and limiting further generation of Treg cells (38). Acquisition of FoxP3 expression by thymocytes coincides with the emergence of phenotypic and functional features of mature peripheral Tregs, including inability to produce IL-2 and emergence of suppressive abilities. Genome-wide analyses of FoxP3 have shown that it binds to ∼700 genes and miRNAs involved in multiple networks, acting both as a repressor and an activator (14, 39); FoxP3 also forms multiprotein complexes with over 360 proteins and acts in cooperation with FoxP1, another member of the FoxP family, in the regulation of gene expression (40, 41).

However, FoxP3 expression alone is not sufficient to establish the full Treg cell phenotype, as indicated by the fact that ectopic expression of FoxP3 fails to induce a large proportion of Treg cell signature genes, and T cell activation in humans transiently induces high levels of FoxP3 but not suppressive abilities (42–44). Thus, in addition to FoxP3 expression, a second process is necessary for the full and stable development of the Treg lineage, and that is the establishment of a specific epigenetic signature (45–47). Tregs in fact exhibit a specific DNA hypomethylation pattern (48) particularly at Treg cell signature genes, such as *FoxP3, CTLA4, IL2ra, and Ikfz2*. Chromatin accessibility of the genes defining Treg cell identity is facilitated by activation of super-enhancers (SE) in Treg precursors, and the genomic organizer Satb1 has a pioneering effect through "preconditioning" of the chromatin landscape and binding to Treg-SE at the early stages of thymic Treg development (17). Interestingly, the *SATB1* locus contains single nucleotide polymorphisms (SNPs) associated with MS (18), suggesting that alterations in the initial events that lead to the generation of Tregs may contribute to genetic susceptibility to immune dysregulation and to disease development. The presence of DNA hypomethylation at Treg signature genes contributes to the maintenance of lineage stability, and does not occur in activated cells which transiently express FoxP3 and which lack suppressive abilities (21).

In addition to Tregs generated in the thymus, peripheral conversion of Treg cells occurs in some organs, such as the colon, where pTregs emerge following encounter with commensal bacteria and their metabolites (49–53), and in the placenta, where they mitigate maternal reactivity to the fetus (54, 55). FoxP3 induction is dependent on the FoxP3 enhancer CNS1, and selective ablation of pTregs in $CNS^{-/-}$ mice induces spontaneous development of pronounced Th2-type inflammation in the gastrointestinal tract and lungs, with concomitant alterations in the composition of the gut microbiota (16, 56).

It has long been known that thymectomy before day 3 after birth induces severe autoimmunity, indicating that pTregs alone are insufficient for the maintenance of cell tolerance (11). Current thinking is that pTregs suppress inflammation at mucosal barriers where they are key contributors to the maintenance of tolerance to a vast array of foreign antigens derived from the microbiota and from the diet, while tTregs control immune responses to self antigens (56, 57). A healthy immune system results from the balanced interaction between the antigenic blast present at barrier surfaces and the active suppression provided by Treg cells. The distinction between pTregs and tTreg is only one of several that can segregate these cells in different subsets, in a graded scale of differentiation and function that we describe below.

Treg Cell Heterogeneity

In humans, all circulating $CD4^+FoxP3^+$ tTreg cells are characterized by high levels of CD25 and low CD127. However, they comprise different phenotypic subsets, which can be separated based on expression of CD45RA and CD25 in naïve ($CD45RA^+CD25^+FoxP3^+$) and effector ($CD45RA^-CD25^+FoxP3^+$) cells; a third subset, which expresses low levels of FoxP3, contains a mixed population of recently activated Tconv cells which do not have suppressive functions and produce cytokines at high levels (44). Naïve Tregs are likely recent thymic emigrants, and accordingly a high fraction express CD31 (58); following activation they differentiate in effector Tregs (44). Effector Tregs express high levels of suppressive molecules, such as CTLA4. A recent study investigating the proteomics and transcriptomics of these subsets has defined both a common Treg and an effector Treg signature, which is maintained even after stimulation and expansion *in vitro*. The main features of the Treg signature are the stability of FoxP3 expression and the inability to produce effector cytokines, the latter characteristic being achieved by the low abundance of transcription factors, such as STAT4, a major activator of IFNγ transcription (59).

The effector and naïve phenotypes are associated with differential expression of other molecules, such as CD39, CCR6, HLA-DR, GITR, LAG3 (60, 61), which are involved in the suppressive function and migratory ability of Tregs, and which may be expressed at different times during the cells' life.

ICOS expression defines two other Treg subsets, and is associated with ability to secrete IL-10 together with TGF-β. $FoxP3^+$ cells receive a different imprinting during thymic development, depending on the presence or absence of costimulation through ICOS and on the thymic antigen presenting cell they interact with (62).

A specialized subset of Treg cells expresses TIGIT, a coinhibitory molecule, and gene profiling of $TIGIT^+$ and $TIGIT^-$ Treg cells indicate that $TIGIT^+$ cells have the attributes of a highly suppressive population specialized in suppression of Th1 and Th17 responses, with increased levels of expression of CTLA4, PD-1, Tim-3, and in humans also of CD39 and CCR6 (63, 64).

Thus, Treg cells can be defined on the basis of the expression of surface markers which identify distinct stages of differentiation and which denote the suppressive competence of each subset.

TREG CELL PLASTICITY

Conventional T cells differentiate into distinct types of effector cells when exposed to particular combinations of cytokines produced by innate immune cells following infection with specific classes of pathogens. The expression of lineage-specific transcription factors and epigenetic modifications underlies the full differentiation of distinct T cell subsets which can optimally deal with the invading pathogens (65). Several Th subsets have been identified to date, and following the initial discovery of the Th1 and Th2 subsets, Th9, Th17, Th22, and Tr1 subsets have been subsequently described. The effector function of these Th subsets mainly consists in the specialized secretion of the most appropriate set of cytokines for each category of challenge, so Th1 cells produce mainly IFNγ in their fight against intracellular bacteria, Th2 cells secrete IL-4, IL-15, and IL-13 to contrast helminths and parasites, and IL-17 produced by Th17 cells protects from fungal infections.

It soon became clear, both in the human and in the murine systems, that also Treg cells have a considerable degree of plasticity, exemplified by the co-expression of multiple transcription factors typical of other CD4 T cell lineages (66). This allows Treg cells to adapt to their location and to be equipped with the surface receptors, effector molecules, and metabolic assets matching those expressed by activated effector cells (**Figure 1**). Indeed, Tregs are able to position themselves in all tissues, wherever immune cells are activated and need to be regulated. Through the vast arrays of chemokine receptors and adhesion molecules they can swarm to non-lymphoid tissues just as conventional T cells do (67), showing that also Tregs have an inherent flexibility which enables adaptation to changing environments and focused immune regulation.

For instance, in response to IFNγ Treg cells express T-bet, which in turn induces expression of CXCR3 and Treg cell accumulation at sites of Th1 mediated inflammation (68), and loss of T-bet-expressing Treg cells causes severe Th1 autoimmunity (69). Relevant to immune reactions occurring in the brain, the CXCR3 chemokine receptor guides cells who express it toward CXCL9, CXCL10, and CXCL11, which are released by both neural and immune cells. Once T cells, both conventional and regulatory, have entered the brain, they are retained by CXCR3 in the perivascular zone rather than spreading in the white matter. This restricts the area of inflammation and favors the interactions between cells, enabling cell contact-dependent mechanisms of immune regulation (70).

Similarly, the microbiota in the intestine induces Treg cells expressing RORγt and STAT3 (71–73), which colocalize with RORγt-expressing Th17 cells. Moreover, high levels of expression of interferon regulatory factor-4 (IRF-4), one of the transcription factors involved in Th2 differentiation, is dependent on FoxP3 expression and is required to efficiently restrain Th2 responses (74). Treg cells can also acquire expression of Bcl6 which induces CXCR5, enabling them to accumulate in germinal centers and to regulate humoral immunity, including B cell affinity maturation and plasma cell differentiation (75).

Although Tregs respond to inflammatory cues and express the master transcriptional factors of Tconv cells, they do not produce the respective cytokines and they maintain stable expression of FoxP3 (56, 76, 77), although it is possible, in conditions of extreme inflammation, that FoxP3 expression is lost and conversion to conventional cytokine-producing cells is reached (78–80). In these cases, "ex-FoxP3" cells acquire the ability to produce IFNγ and are pathogenic in the experimental model of MS, experimental autoimmune encephalomyelitis (EAE) (78).

TREG CELL MECHANISMS OF ACTION

To—literally—gain a picture of Treg's mode of action, a good starting point is the definition of their tissue distribution and, more microscopically, of their positioning relative to the other immune cells during immune responses. tTregs circulate in the periphery and populate peripheral lymph nodes, where at the steady state they constitute 10–15% of the CD4 cell pool (81, 82). Here, Tregs aggregate in clusters with activated autoreactive effector CD4 T cells, which they can effectively "govern" given their close proximity, thus suppressing incipient autoimmunity (83). An interesting study showed that the first responders to the IL-2 produced in the initial phase of the immune responses are $STAT5^+$ Treg cells, which are promptly activated and form a "safety net" constraining subsequent activation of conventional T cells (84). Indeed, Treg express very high levels of CD25, which enables them to capture IL-2, thereby depriving conv T cells of the primary cytokine which sustains their proliferation, and inducing apoptosis (85). This ability has earned Treg cells the epithet "IL-2 sink," although subsequent studies challenge the notion of IL-2 deprivation as a prominent mechanism of suppression, at least *in vivo* (86).

Once they are positioned in close proximity of the cells they need to regulate, Tregs use different molecules to oppose Tconv activation, the most prominent of which is CTLA4, which captures its ligands CD80 and CD86 thus making them unavailable for binding to CD28 on Tconv cells (87). Moreover, CTLA4 has a higher affinity for CD80 and CD86 than does CD28, thus $CTLA4^+$ Tregs outcompete CD28-expressing T conv cells for interaction with Dendritic Cells (DCs) (88). Also, CTLA4 induces upregulation of the tryptophan catabolizing enzyme indoleamine2,3-dioxygenase (IDO) by DC, inducing their regulatory phenotype (89). This modulation of antigen presenting cell maturation and function represents a major mechanism used by Treg cells to suppress immune responses.

Endangered or dying cells release potent proinflammatory stimuli, such as ATP, which Treg cells are able to catabolize through the ectoenzymes CD39 and CD73, which degrade ATP to AMP and adenosine (90, 91). Nucleotide catabolism achieves dampening of the immune response through both the removal of an inflammatory molecule (ATP) and the generation of highly immunosuppressive adenosine, which can bind to its receptors expressed on the surface of all immune cells (92, 93).

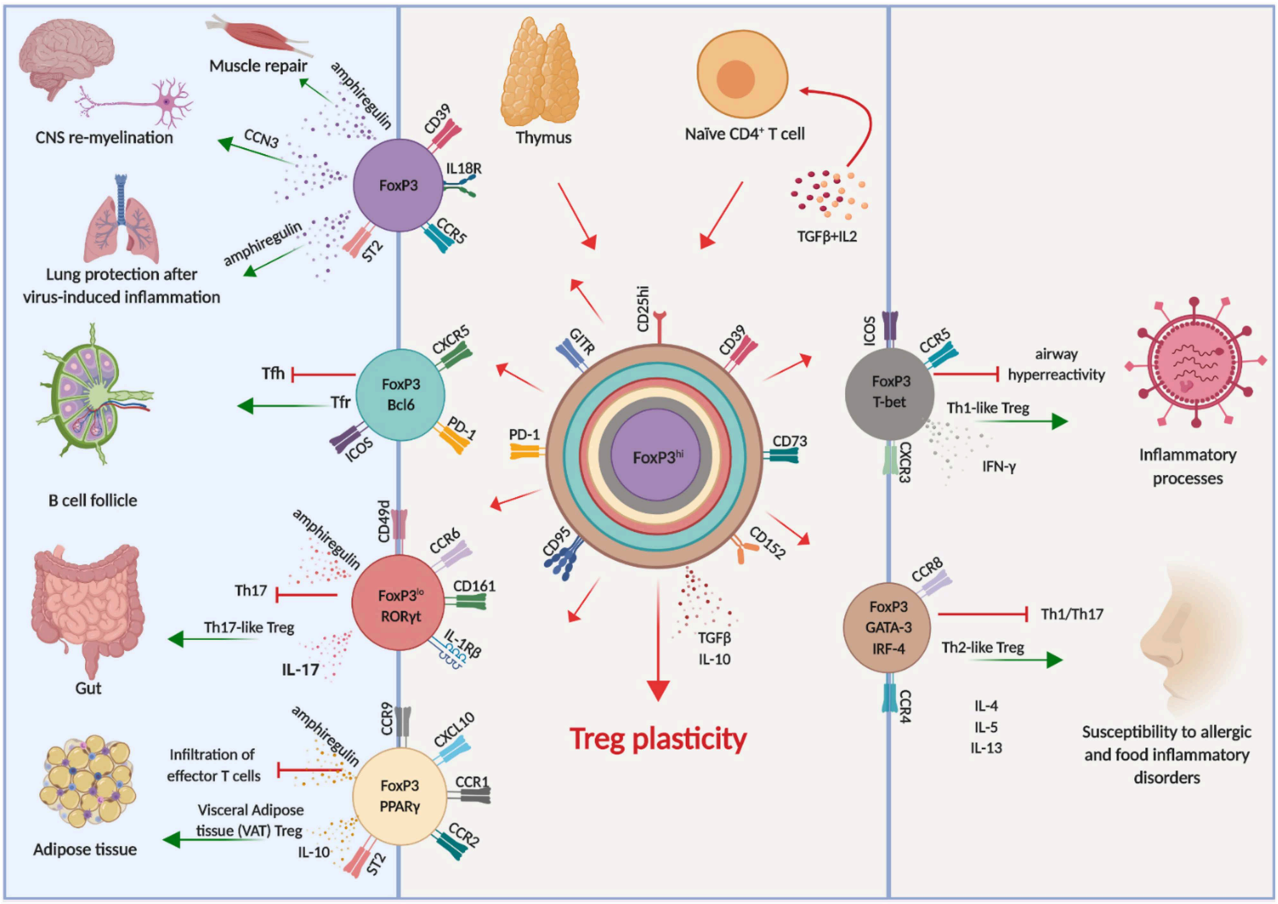

FIGURE 1 | Treg cell plasticity. Treg cells have the potential to acquire expression of the molecular machinery which enables them to adapt to different environments, shadowing effector T cells and tissue-resident cells. This potential is schematically illustrated by the different colors, each corresponding to distinct cellular localization. Arrows indicate the known pathways of functional differentiation of Tregs and the tissues and organs where they can localize.

CD39 expression identifies Tregs with an effector-memory phenotype, and these cells are more stable and present increased functionality (94). This pathway is of great interest as in addition to a general downregulation of aspecific inflammation, it also impacts on the generation of pathogenic Th17 cells. ATP is a potent activator of the inflammasome, with consequent release of the proinflammatory cytokine IL1β (95), and it also induces IL-23 release by a subset of DCs (96), two cytokines involved in Th17 differentiation. Moreover, ATP is directly involved in the generation of Th17 cells in the intestine (97), the major site of antigenic challenge and consequent cell activation. Thus, $CD39^+$ Tregs cells at sites of inflammation may contrast the generation of pathogenic effectors. Interestingly, CD39 expression can also be induced in Th17 cells conferring immune-suppressive properties (98), and during the resolution of inflammation, Th17 cells can transdifferentiate into Tregs (99), with CD39 expression correlating with the ability to produce IL-10 (100). Thus, Th17 and Treg cells' functions intersect also in this pathway, and in this regard it is interesting to notice that while human Treg cells only express CD73 at minimal levels, if any, Th17 in inflammatory sites have high levels of CD73 on their surface (101); thus, the generation of immune-suppressive adenosine through the concerted action of CD39 and CD73 may occur when Th17 and Treg cells are in close proximity, as happens in sites of immune regulation (83).

Other mechanisms of action include the release of immune-suppressive cytokines, such as TGF-β (102), IL-10 (103), IL-35 (104), and of cytotoxic molecules, such as perforin (105), granzyme (106), and cAMP (107, 108).

Treg cells may also acquire tissue-specific abilities, not limited to immune suppression. Tregs stably express high levels of the enzyme 15-hydroxyprostaglandin dehydrogenase, particularly in the visceral adipose tissue, which enables them to catabolize PGE2 into 15-keto-PGE2, which in turn inhibits the proliferation of $CD4^+$ Tconv cells (109). In the muscle, Tregs participate to tissue repair through the secretion of amphiregulin, which facilitates the regeneration of muscle satellite cells (110); similarly, amphiregulin production by Treg cells helps contain the damage to lung tissue following infection (111).

In the CNS, in a mouse model of lysolecithine-induced focal demyelination, Treg cells have been shown promote oligodendrocyte differentiation and remyelination through the release of CCN3, a growth-regulatory protein usually expressed in the developing brain (112). Moreover, ablation of Treg cells during acute spinal cord injury delays tissue remodeling (113).

The skin, another barrier site constantly exposed to exogenous antigens, hosts significant numbers of Treg cells (114), although curiously these are mainly represented by tTregs which colonize this tissue shortly after birth (115). Skin Treg have been shown to facilitate cutaneous wound healing and epithelial stem cell differentiation through expression of Jagged 1 (116), and to regulate fibroblast activation and fibrosis through expression of a skewed transcriptional programme dominated by GATA3 (117).

Thus, Treg cells can adopt several mechanisms for the down modulation of immune responses at both in lymphoid and non-lymphoid tissues, which generally safeguard against pathological and autoimmune reactions.

TREGS AND THE MICROBIOME

In the words of Blaser, the microbiota is at the same time self and non-self, being part of our biology but rapidly changing in response to external stimuli (118). While tolerance to self-antigens is instructed in the thymus, post-thymic education to tolerate foreign antigens occurs in the intestine, which is colonized by trillions of microbes with whom a healthy symbiotic relationship is established. The immune system shapes and preserves the ecology of the microbiota, which in turn tunes and calibrates immune cells, in a continuous homeostatic dialogue (119, 120). Changes in diet, improved sanitation, and mindless use of antibiotics have had a profound impact on the microbiota composition of contemporary populations in western countries, reducing diversity and promoting enrichment with bacterial strains capable of inducing inflammation, mainly through the generation of Th17 cells, but also through decreased induction of suppressive Tregs. These changes in microbiota composition are thought to contribute significantly to the increase in autoimmune and inflammatory diseases observed in recent times (2, 121).

Gut Tregs, which represent a large proportion of mucosal T cells interacting with the commensal microbes, are generated both through the peripheral conversion of Tconv cells in response to the microbiota (49, 51, 52, 56, 73), or following colonization and expansion of tTreg (50, 122–124), thus establishing tolerance, and actually also influencing the composition of the microbiota: Treg cell deficiency is accompanied by gut dysbiosis (125), and impaired pTreg generation leads to perturbations in the composition and metabolic function of intestinal microbiota (126).

Metabolites produced by the commensal bacteria also influence Tregs. For instance, short chain fatty acids (SCFA) including butyric and propionic acid induce the differentiation of Treg cells through H3 acetylation at the CNS3 and CNS1 regions of the FoxP3 gene locus (49, 127). Polysaccharide A (PSA) produced by the commensal *Bacteroides fragilis* prevents intestinal inflammatory disease and induces the generation of IL-10-producing FoxP3$^+$ Treg cells (128–130). These expanded Treg populations express CD39, which in addition to its role in catabolizing ATP, also modulates their migratory capacity (131).

The microbiota consists also of organisms that direct pro-inflammatory immune responses and Th17 differentiation. Th17 are essential for the protection against extracellular bacteria and fungi (132–134), but their pathogenic role in inflammatory disease is also well documented. Commensal microbiota are involved in the development of intestinal Th17, with *Candida albicans* playing a prominent role (132) followed by segmented filamentous bacteria (SFB), which promote Th17 differentiation and brain inflammation (135, 136).

The picture that emerges is that the composition of a healthy microbiome is not accidental, and was established after millennia of coevolution. The balance between the generation of proinflammatory effector cells, which combat potentially pathogenic microbes, and immune-suppressive regulatory T cells, which oppose and downmodulate these responses, is an active process. Changes in this balance, either due to an altered composition of the microbiota or to an intrinsic defect of Tregs, may generate inflammatory responses that target organs distant from the gut, as the brain (**Figure 2**).

NEUROIMMUNOLOGY: MULTIPLE SCLEROSIS

Multiple sclerosis is an inflammatory disease of the central nervous system characterized by recurrent attacks of neurological dysfunction from which early on in the disease course patients gradually recover; in most patients, a phase characterized by progressive clinical worsening then ensues, and permanent disability is established (137–139). Pathologically, MS is characterized by the appearance of demyelinating lesions with extensive inflammatory infiltrates both in the white and in the cortical gray matter, pointing to an involvement of the immune system in disease pathogenesis. Indeed, genome-wide association studies (GWAS) have indicated that virtually all MS-associated genes are involved in immune processes (18, 140, 141), with expression of the HLA-DRB1*15:01 allele being the most dominant risk factor. This genetic association argues strongly in favor of the central role of the adaptive immune response in MS, since T cell activation sparks from the interaction of TCRs with HLA molecules.

MS arises for an unfortunate combination of factors and events, and different risk factors have been identified. The genetic component is evidenced by a high concordance rate in monozygotic twins (25–30%), but the fact that in this case the relative risk does not reach 100% points to a role of environmental factors in inducing disease. Low vitamin D levels, cigarette smoking, the presence of environmental toxins, obesity, and infection with EBV virus are the most consistent environmental factors linked to MS (142, 143). In more recent years, the influences of the microbiome in inducing or preventing MS have emerged. Alterations in the composition of the microbiome in contemporary populations is thought to be partly

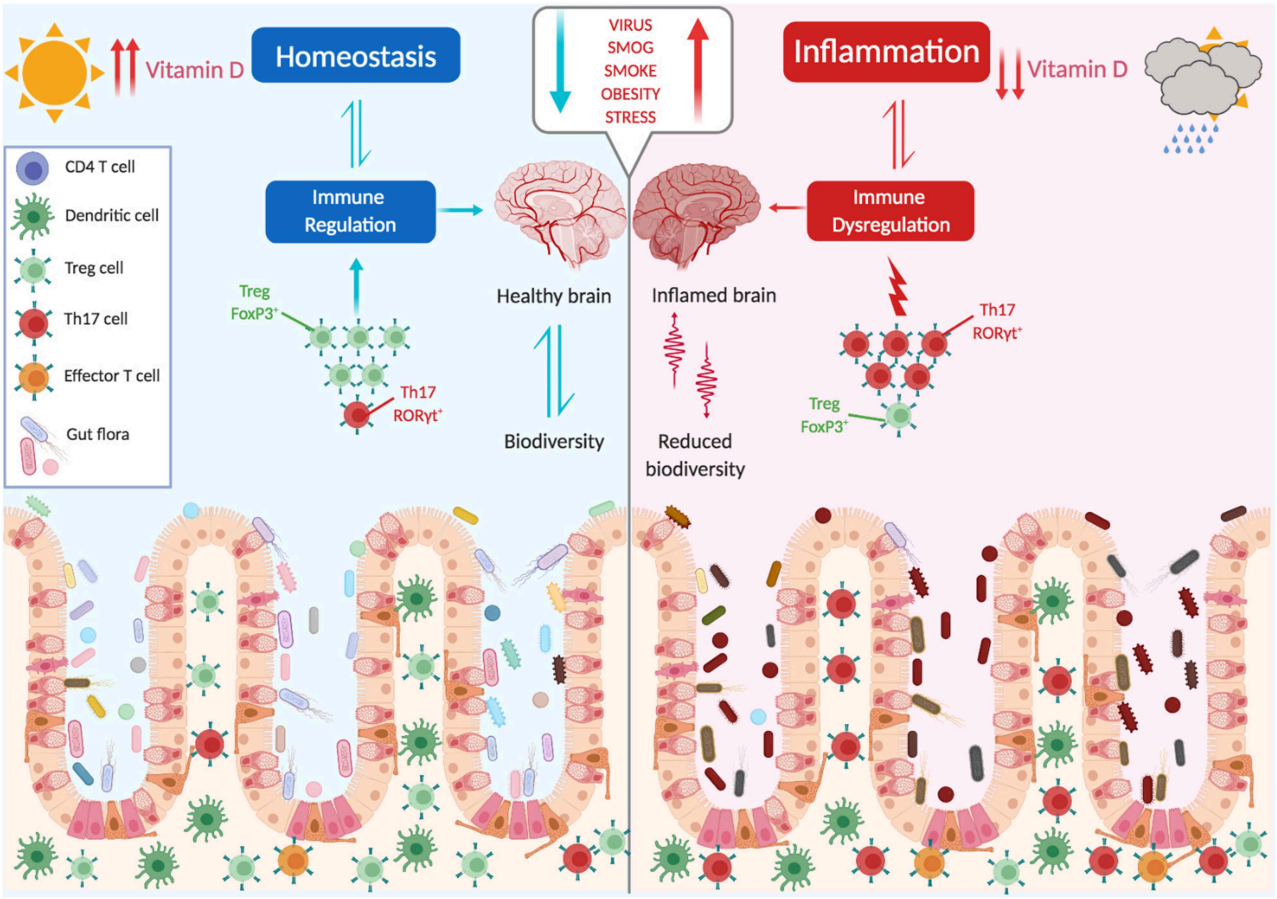

FIGURE 2 | The microbiota shapes immune responses. The balance between proinflammatory and immune suppressive factors (dysbiosis, vit. D, stress, obesity, smoking, infections, diet) is critical for the maintenance of tolerance and avoidance of autoimmunity. Several environmental factors can influence this balance, with a main role being played by the gut microbiota.

responsible for increases in autoimmune disorders including MS. An interesting paper by Vatanen et al. (144) showed that depending on the composition of the microbiota, LPS derived from different strains has different immunogenicity and may lead to altered immune maturation and predispose to autoimmunity. Indeed, the intestinal microbiome of MS patients shows distinct changes compared to healthy controls, particularly with a significant reduction in biodiversity (145–148). Interestingly, fecal transplants from MS patients in transgenic models of brain autoimmunity precipitate disease (149, 150), further supporting a role of microbial composition in inducing pathological immune responses.

Dysbiosis, with a shift in the microbiota composition toward a proinflammatory asset, also determines alterations in intestinal permeability, granting access of bacterial components and preceding the onset of brain autoimmunity in the experimental model (151, 152).

The CNS is kept separate from the rest of the organism and from the outside environment by several anatomical and cellular layers, in order to protect the resident cells which are particularly vulnerable to immune-mediated damage and unable to regenerate. The meninges surround the brain and contain the cerebrospinal fluid, and are also host to a functional lymphatic system which enables drainage from the CNS to the deep cervical lymph nodes (153, 154). Although it is equipped with a lymphatic system, the brain is an immunologically unique site, and it is shielded from the blood circulation by a specialized vasculature composed by endothelial cells joined together by tight junctions and surrounded by glial podocytes, thus creating a nearly impermeable boundary, the blood brain barrier (BBB) (155), which represents a limiting factor to the entry of metabolites and of immune cells into the CNS. Alterations of the permeability of the BBB clinically correspond to the formation of the typical acute gadolinium-enhancing regions visible by magnetic resonance imaging (MRI) scans. Interestingly, the BBB is vulnerable to changes in the gut microbiota (156), and coloniziation of mice with a single organism, *B. fragilis*, can restore permeability (157).

Activated and memory T cells in the periphery express the adhesion molecules and chemokine receptors which enable them to cross the BBB and to infiltrate the brain tissue (158, 159), and this feature is exemplified by the induction of neuroinflammation

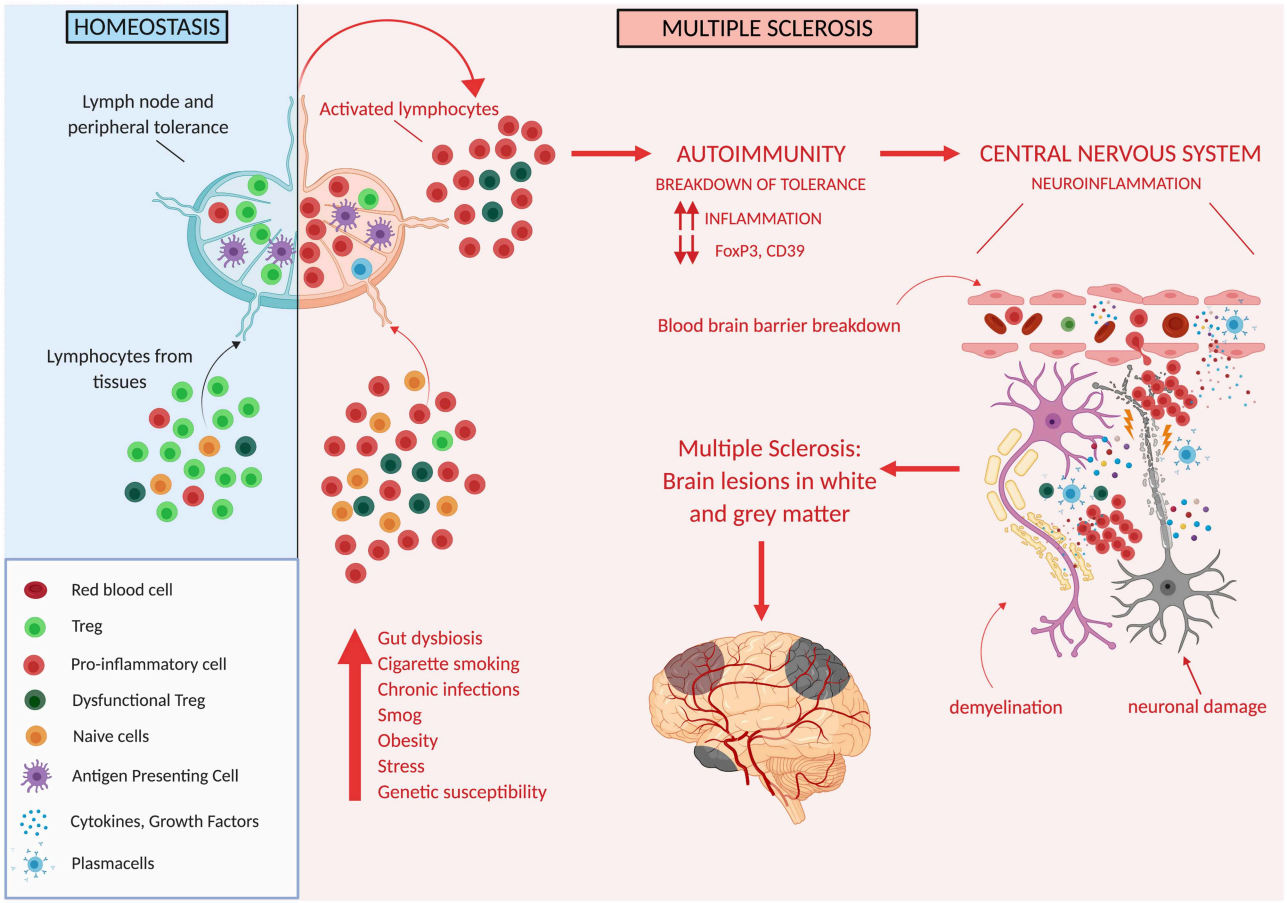

FIGURE 3 | Tregs in multiple sclerosis. Genetic and/or environmental factors may induce hordes of proinflammatory cells and thus may overcome the ability of Treg cells to suppress inflammation. This may unleash autoreactive immune cells which cross the blood brain barrier and target different areas of the CNS ultimately determining the development of white and gray matter lesions.

in the murine model through the transfer of myelin-specific T cells which upon injection in naïve mice rapidly accumulate in the brain, generating the typical lesions similar to those observed in MS patients. CCR6 expression has been shown to be a requirement for migration in the CNS (160), following a gradient of CCL20 produced by the choroid plexus, which allows entry in the brain ventricles. Once in the tissue, these cells can become reactivated following interaction with resident antigen-presenting cells (APCs), such as microglia or myeloid cells, and can start producing inflammatory cytokines which increase BBB permeability and initiate the cascade of infiltration by pathogenic proinflammatory cells, and thus the typical perivascular infiltrates are formed (161). $CD8^+$ T cells also gain access to the CNS, and actually constitute the majority of the T cell infiltrate; through the release of cytotoxic molecules, such as perforin and granzyme they induce demyelination (138, 162, 163), and they have also been shown to produce IL-17 (163). "Special" CD8 T cells also participate to disease: mucosal-associated invariant T (MAIT) cells (164, 165), a subset of innate-like T cells which recognize molecules derived from bacterial metabolism and which usually reside in the gut, where they control the intestinal microbiome. These cells have been found in the infiltrating lesions of MS patients, where they produce IL-17. Also B cells have a pathogenic role in MS (166): the presence of oligoclonal bands is a hallmark of the disease, and ectopic B cell follicles are present in meninges from patients with MS (167, 168). Moreover, the spectacular results obtained in clinical trials using antibodies targeting B cells confirm the role that these cells play in the disease, including their role as APC which may reactivate autoreactive T cells crossing the BBB (169–171).

Together with these pathogenic cells, also Tregs gain access to the CNS.

TREG CELLS IN MULTIPLE SCLEROSIS

The CNS is an excellent organ to study in models of inflammatory autoimmune disease, since physiologically it is free from activated immune cells, such as lymphocytes and macrophages. The induction of an autoimmune reaction directed against CNS components—typically myelin antigens—is a well-established

model for human multiple sclerosis, and has provided crucial insight on the immune mechanisms underlying the disease.

The initial studies on Treg cells in MS were those performed in the murine model of brain autoimmunity, where it was discovered that their ablation aggravated disease, while adoptive transfer inhibited it (172, 173). Several studies then addressed the issue of Tregs in MS patients, which showed that Treg cell frequency as a whole, mostly determined by the gross measurement of $CD4^+CD25^{high}$ circulating cells, was shown to be in the physiological range (174–177), or decreased (178, 179). However, investigations on the functional abilities of these cells with the identification of distinct subsets have revealed impairments at different levels which partly explain the defects in immunoregulation underlying the autoimmune attack in MS (180–182).

It has been shown that the frequency in the peripheral blood of Tregs expressing CD39 is reduced in MS patients, and that this correlates with a lower ability to catabolize ATP (90). Interestingly, $CD39^+$ Treg cells are able to efficiently suppress IL-17 production by Th17 cells, contrary to their $CD39^-$ counterpart, thus their reduction in MS patients may participate to the ineffective control of Th17-driven inflammation in this disease (183). A recent study has shown that in MS patients experiencing clinical relapses the frequency of $CD39^+$ Treg cells was increased (184), possibly in an attempt to counterbalance active inflammation (185). In the murine model, PSA produced by the human intestinal commensal *Bacteroides fragilis*, induces expansion of $CD39^+$Treg cells with protective effects on EAE manifestations, and ablation of $CD39^+$ abrogates these effects (186).

Treg cells from MS patients have been shown to express lower levels of FoxP3 (187), and a more detailed study later showed that in patients there is prevalent expression of the FoxP3 isoform lacking exon2, making Treg cells less efficient in inhibiting the development of proinflammatory cells (28).

Genetic polymorphisms in Treg-relevant genes may also underlie the development of MS. For example, a high risk of MS is associated with polymorphism in CD25 (188).

Also resident cells of the CNS can influence Treg cell activity. Neurons, for instance, produce TGF-β and might directly affect T cells. One study has shown that neurons participate to the conversion of encephalitogenic CD4 T cells to Treg cells expressing FoxP3 and mediating suppression through CTLA4 (189). Moreover, serotonin, a CNS neurotransmitter, favors the expansion *in vitro* of Treg cells from MS patients while reducing production of IL-17 by Tconv cells, suggesting that it acts as a neuroprotectant in the attempt of resolving inflammation (190).

Adding to all this, another aspect is resistance of Tconv to the suppressive action of Treg cells: effector T cells may "break free" and escape Treg suppression, mainly through accelerated production of IL-6 and STAT-3 signaling, as has been shown to happen in MS patients (191–193).

Thus, Treg impairment at different levels has been described to occur in MS patients, and likely plays an important role in determining altered immune regulation and disease susceptibility (**Figure 3**).

EFFECTS OF IMMUNOMODULATORY DRUGS FOR MS ON TREGS

Although there is no cure for MS, 15 drugs are now available for modifying disease course of the relapsing-remitting form (194, 195). Early treatment is recommended in order to achieve the goal of "no evidence of disease activity" (NEDA) (196), and currently no patient is left without therapy. All these treatments target the immune system, and most also exert an effect on Treg cells.

The first injectable drugs approved for use in MS were type I interferons and glatiramer acetate (GA), and they are used as first-line agents to slow disease progression. Type I interferons have a plethora of immunomodulatory functions, in addition to their role in viral interference (197, 198). In MS patients, treatment with this drug enhances regulatory cell function and increases FoxP3 mRNA levels, thus favoring immune suppression (199–201). GA is a synthetic polymer of aminoacids, whose complex mechanism of action are still incompletely understood, and involves both immunomodulatory and neuroprotective effects. Patients treated with GA show increased levels of FoxP3 (202) and of Treg function (203), seemingly through a direct effect on thymic output of naïve Tregs.

Natalizumab is a humanized monoclonal antibody directed against the α4 chain of α4β1 integrin (CD49d), and it interferes with lymphocyte migration in the CNS. It is highly effective in reducing the annual relapse rate (204, 205), although it may predispose to developing opportunistic infections in the CNS. Interestingly, contrary to Tconv cells Tregs express very low levels of CD49d (206) thus their migration to the CNS may not be affected by therapy with natalizumab. Indeed, given the low expression of CD49d by Treg cell, the therapeutic effects of this drug seem to be independent of a direct effect on Tregs (207).

Fingolimod was the first oral drug to be approved for use in MS. It is a $S1P_1$ antagonist, thus it prevents T cells from exiting the lymph nodes, with the result of reduced numbers of circulating T cells (208, 209). However, it is now clear that the effects of fingolimod on immune cells go beyond the modulation of their migratory properties. In Treg cells $S1P_1$ inhibition leads to increased thymic egress and suppressive function, through blockade of the Akt-mTOR pathway (210). Deletion of $S1P_1$ in a mouse model induced development of systemic autoimmunity, and acute $S1P_1$ ablation enhanced susceptibility to EAE (211). In the same study it was also shown that in patients undergoing treatment with fingolimod the phenotypic conversion from naïve Treg cells to effector T cells occurs with greater frequency. Fingolimod also affects Treg plasticity by reducing the expression of T-bet and of IFNγ and by enhancing expression of Tim-3, a marker associated with superior suppressor capacity (212). In general, these studies show that Treg function is increased following treatment with fingolimod.

Dimethyl fumarate (DMF) is an oral drug which was initially used to treat psoriasis, and was later approved for use in MS (213). The mechanism of action is still not completely understood, but seems to rely on the activation of the nuclear-related factor 2 (Nrf2) antioxidative response pathways (214). Very few studies have addressed the impact of DMF directly on Treg cells, and

besides an increase of FoxP3$^+$ cells during the first months of treatment (215), no other effect has been described.

Alemtuzumab is a humanized monoclonal antibody specific for CD52, a protein highly expressed by T and B cells and at lower levels on innate immune cells. Two trials established its safety and efficacy in MS patients (216, 217). Treatment with alemtuzumab induces long-lasting lymphopenia, and the beneficial effect on MS seems to be due on the re-equilibration of the immune system which occurs through depletion and repopulation of lymphocytes, which includes an enrichment in Treg cells (218, 219), and an increase of Treg suppressive function (220).

Cladribine (CdA) is a synthetic deoxyadenosine analog. Like deoxyadenosine, it enters lymphocytes through an efficient transport mechanism. Once in the cell, deoxyadenosine has two potential fates: irreversible deamination by adeonine deaminase (ADA), leading ultimately to the uric acid excretion pathway, or serial phosphorylation by deoxycytidine kinase (DCK) to dATP. Cladribine, on the other hand, is resistant to deamination by ADA because of its chlorinated purine ring structure and thus can only phosphorylated by DCK to its lymphocytotoxic form, 2-chlorodeoxyadenosine triphosphate, which accumulates and is incorporated into DNA. This results in DNA strand breakage and inhibition of DNA synthesis and repair, and to cell death. The selective toxicity of CdA toward both dividing and resting lymphocytes makes the drug useful as an antileukemic or immunosuppressive agent, and indeed it is used in hematologic cancers, such as B cell chronic leukemia and particularly hairy cell leukemia (221, 222). Subsequently, the drug was tested for treatment of MS. The "CLARITY" trial for relapsing remitting MS showed a significant reduction in relapse rate (223) and the drug is now approved for treatment of "rapidly evolving severe" MS (defined as at least two relapses in the previous year and an MRI scan showing new, or bigger, lesions). The beneficial effects of cladribine seem to be achieved through targeted and sustained reduction of circulating T and B lymphocytes, but it is possible that additional immunomodulatory actions participate to its effects (224). MS patients that express low levels of CD39 are likely to respond to cladribine therapy, since they lack the level of immune-regulation provided by the generation of suppressive adenosine, and thus may benefit from pharmacologically induced increases in adenosine.

From this survey of the effects of currently used immunomodulatory drugs it emerges that the most frequently measured effect is an increase in frequency and in suppressive function of Tregs, and although these cells are not the direct target of these therapies, nonetheless the improved immune regulation they provide contributes to altering the progression of the disease.

CONCLUSIONS AND FUTURE DIRECTIONS

The Treg cell population is vastly heterogeneous, as most complex makings of Nature, and comprises an army of cells expressing graded levels of transcription factors and of effector molecules. FoxP3 dominates the machinery of these cells, and through the interactions with both the genes made accessible by epigenetic mechanisms and with the nuclear proteins available for binding, it regulates transcription of the quintessential Treg molecules. Treg differentiation is a plastic and actively maintained state determined by the collective activity of the whole transcriptional network, and this complexity confers elasticity and adaptability to changing environments. Treg cells swarm to areas of immune interrogation and interact with APCs in lymphoid tissues, as do naïve Tconv cells, but they can just as easily migrate in tissues together with T effector cell populations, responding to the same cues; moreover, besides adapting to the environment and downregulating the immune response, Tregs provide molecules which sustain tissue cells, actively participating to tissue protection and regeneration. One prominent site of Treg activity is the intestine, where these cells modulate immune interactions with the microbiota and help maintain tolerance. Changes in microbiota composition in present-day populations, with a prominent representation of proinflammatory microorganisms and a significant reduction of biodiversity, have been shown to underlie the steep increase of autoimmune and allergic disorders observed in western countries, raising the level of inflammation to a point where Treg cells are unable to contrast the fire. It is also possible that subtle defects in Treg function may be unmasked by excessive inflammation, and this may explain why only some individuals succumb to autoimmunity.

Many themes still need to be investigated. For instance, in recent years the role of circadian factors on immune cell function has emerged as a factor which also impacts the development of autoimmunity. Indeed, immune responses have a rhythmicity regulated by intrinsic timers (225, 226), and in animal models susceptibility to sepsis or to EAE induction are significantly and intriguingly dependent on the time of the day (227–230). Only few studies have addressed the issue of circadian rhythms on the functions of Treg cells (231–234), all suggesting that also Treg cells are under circadian control. Also the recently identified resolvins, molecules which mediate resolution of inflammation (235), are a fascinating area of investigation and have been shown to induce Treg cell polarization in humans (236) flipping the balance of immune reactions toward immune regulation and away from inflammation. Additionally, as mentioned, T cell identity is maintained not only by transcriptional programs, and post-transcriptional processes are in place to rapidly tune cell fate decisions in response to changing environments. Non-coding RNAs are RNA transcripts which do not code for protein products but instead regulate gene expression by driving post-transcriptional repression through pairing with mRNA. They include microRNA, small interfering RNA (siRNA), long non-coding RNA, and the recently discovered circular RNA (237–239). The finding that miRNA disruption selectively in Tregs leads to fatal systemic autoimmunity similar to the disease occurring in FoxP3 deficient mice indicates that non-coding mRNAs are central for maintaining Treg cell identity and function (25, 26). Indeed, studies on miRNA and on their role in Treg cell biology have identified a miRNA Treg signature which seems to be conserved across species (240, 241). miRNA are also involved in the pathogenesis of autoimmune diseases, including

MS, mainly through the modulation of the Treg/Th17 balance (242–245), and are interestingly found in the extracellular vesicles released by activated lymphocytes. Moreover, they may directly participate to Treg-mediated immune suppression (246).

These areas of investigation may hold the answer to what goes wrong in CNS autoimmunity, although given the complexity of the human meta-organism and of its immune network we have many more than one question, closer to one hundred thousand.

AUTHOR CONTRIBUTIONS

GB and MS wrote the manuscript. FG and GG revised the literature. MS and GB conceived and edited the figures. All authors contributed to manuscript revision.

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TNFα-Signaling Modulates the Kinase Activity of Human Effector Treg and Regulates IL-17A Expression

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Maintenance of regulatory T cells $CD4^+CD25^{high}FOXP3^+$ (Treg) stability is vital for proper Treg function and controlling the immune equilibrium. Treg cells are heterogeneous and can reveal plasticity, exemplified by their potential to express IL-17A. TNFα-TNFR2 signaling controls IL-17A expression in conventional T cells via the anti-inflammatory ubiquitin-editing and kinase activity regulating enzyme *TNFAIP3*/A20 (tumor necrosis factor-alpha-induced protein 3). To obtain a molecular understanding of TNFα signaling on IL-17 expression in the human effector ($_{eff}$Treg, $CD25^{high}CD45RA^-$) Treg subset, we here studied the kinome activity regulation by TNFα signaling. Using FACS-sorted naïve ($_{naïve}$Treg, $CD25^{high}CD45RA^+$) and $_{eff}$Treg subsets, we demonstrated a reciprocal relationship between TNFα and IL-17A expression; $_{eff}$Treg ($TNFα^{low}$/$IL\text{-}17A^{high}$) and $_{naïve}$Treg ($TNFα^{high}$/$IL\text{-}17A^{low}$). In $_{eff}$Treg, TNFα-TNFR2 signaling prevented IL-17A expression, whereas inhibition of TNFα signaling by clinically applied anti-TNF antibodies led to increased IL-17A expression. Inhibition of TNFα signaling led to reduced *TNFAIP3* expression, which, by using siRNA inhibition of *TNFAIP3*, appeared causally linked to increased IL-17A expression in $_{eff}$Treg. Kinome activity screening of CD3/CD28-activated $_{eff}$Treg revealed that anti-TNF-mediated neutralization led to increased kinase activity. STRING association analysis revealed that the TNF suppression $_{eff}$Treg kinase activity network was strongly associated with kinases involved in TCR, JAK, MAPK, and PKC pathway signaling. Small-molecule-based inhibition of TCR and JAK pathways prevented the IL-17 expression in $_{eff}$Treg. Together, these findings stress the importance of TNF-TNFR2 in regulating the kinase architecture of antigen-activated $_{eff}$Treg and controlling IL-17 expression of the human Treg. These findings might be relevant for optimizing anti-TNF-based therapy and may aid in preventing Treg plasticity in case of Treg-based cell therapy.

Keywords: Treg, FOXP3, TNF, anti-TNF, IL-17A, JAK, TCR

HIGHLIGHTS

- Naïve and effector $CD4^+$ regulatory T cells have a reciprocal IL-17A–TNFα relationship; $_{eff}$Treg (TNF^{low}/ IL-$17A^{high}$) and $_{naïve}$Treg (TNF^{high}/ IL-$17A^{low}$).
- TNFα-TNF receptor-2 signaling regulates IL-17A expression via ubiquitin-editing *TNFAIP3*/A20 protein in $_{eff}$Treg.
- TNFα suppresses T-cell receptor and Janus kinase protein activity and promotes IL-17A expression in $_{eff}$Treg.
- siRNA-mediated *TNFAIP3* inhibition of $_{eff}$Treg, similar to TNFα signaling inhibition by anti-TNF treatment, leads to enhanced *IL17A* expression.
- TNFα signaling regulates the kinase architecture of antigen-activated $_{eff}$Treg.

INTRODUCTION

Regulatory $CD4^+CD25^{high}FOXP3^+$ T cells (Treg) are essential for human immune homeostasis (1). Human Treg cells reveal heterogeneity and contain multiple cell subsets that are characterized by differential expression of maturation, activation, and migration markers (2). At birth, the majority of the Treg are naïve (3), while later in life, the frequencies of $CD45RA^-$ memory (effector) Treg increase at the expense of naïve Treg frequencies (4). Naïve ($_{naïve}$Treg) and effector ($_{eff}$Treg) Treg have distinct transcriptional, proteomic, metabolic, as well as enhancer and promoter landscapes (5–7).

Effector Treg cells were shown to express pro-inflammatory cytokines such as the autoimmune associated pro-inflammatory cytokine IL-17A, but also naïve Treg was found to produce IL-17A albeit at lower frequencies (5, 8). IL-17A-producing Treg have been observed in human inflammatory diseases such as psoriasis and IBD, suggesting that they contribute to the inflammatory process as has been demonstrated in mouse models (9–14). Although some cues that regulate IL-17A expression by Treg have been identified, including mTOR inhibition (15), CD28 superagonist stimulation (16), and platelet microparticle interaction (17), our mechanistic understanding of IL-17A expression by Treg is limited, let alone that this information is available for naïve and effector Treg. Recently, it has been elucidated that TNFR2 signaling is vital to establish Treg stability by promoting FOXP3 expression and inhibiting secretion of pro-inflammatory cytokines like IL-17A and IFNγ (18, 19). In conventional CD4+ memory T cells, inhibition of TNFR2 signaling by anti-TNF led to reduced expression of the anti-inflammatory regulator tumor necrosis factor-alpha-induced protein 3 (*TNFAIP3*, also known as A20), and as a consequence, this resulted in increased IL-17A expression (20). *TNFAIP3*/A20 acts as a ubiquitin-editing enzyme that regulates multiple other signaling pathways such as IL-17R (21) signaling and kinase activity [e.g., PKC (22), TCR (23), and MAPK (24)].

TNF-TNFR2 signaling appears essential for human Treg expansion and proper function and additionally an autologous TNFα signaling feedback loop has been proposed that regulates IL-17A expression in human Treg (18, 19, 25–29). Anti-TNF therapy is successfully used for the treatment of severe chronic inflammatory diseases such as inflammatory bowel diseases, psoriasis, psoriatic arthritis, and rheumatoid arthritis (30–33). Paradoxically, it has been observed that in 0.6–5% of the patients treated with anti-TNF medication, this might unintentionally trigger specific forms of immune pathology, suggesting that inhibition of anti-TNF therapy affects Treg function (34–37). If and how naïve and effector Treg are affected by inhibition of TNFα is not known.

We hypothesize that TNFα signaling controls IL-17A expression in Treg by interfering at the level of kinase activity, which we here explored in $_{eff}$Treg. We demonstrate that inhibition of TNFα signaling by anti-TNF *in vitro* led to increased IL-17A expression. Down-regulation of the anti-inflammatory mediator *TNFAIP3* played a role in this process. Comprehensive kinome analysis revealed that inhibition of TNFα signaling in $_{eff}$Treg unexpectedly led to an increase of a kinase activity network containing TCR-linked kinases and immune signaling pathway such as the JAK. Small-molecule-based inhibition of these pathways prevented the anti-TNF-induced IL-17A expression in $_{eff}$Treg.

RESULTS

$_{naïve}$Treg and $_{eff}$Treg Cells Reveal a Reciprocal IL-17A—TNFα Relationship

To investigate the link between TNFα and IL-17A expression in naïve and effector Treg, FACS-sorted $_{naïve}$Treg ($CD4^+CD45RA^+CD25^+$) and $_{eff}$Treg ($CD4^+CD45RA^-CD25^{high}$) (**Figure 1A**) derived from healthy volunteers were stimulated with PMA plus ionomycin, and subsequently *TNFA*, *IL17A*, *IL17F*, and *RORC* (RORγt) expression was accessed by RT-qPCR (**Figure 1B**). As compared to $_{eff}$Treg, $_{naïve}$Treg expressed significantly lower levels of *IL17A*, *IL17F*, *and RORC* ($p = 0.0005$, $p = 0.0093$, and $p = 0.0016$, respectively), while *TNFA* expression was higher ($p = 0.0002$) (**Figure 1B**). Next, we compared the fold change in gene expression between the Treg subsets and observed a reciprocal gene expression signature for *TNFA*, *IL17A*, *IL17F*, and *RORC* (**Figure 1C**). Correlation analysis revealed a reciprocal relationship between *TNFA* and *IL17A* ($r = -0.50$), *IL17F* ($r = -0.42$), and *RORC* ($r = -0.68$) (**Figure 1D**). As expected, a strong positive correlation between IL17A/IL17F ($r = 0.81$), IL17A/RORC ($r = 0.74$), and IL17A/RORC ($r = 0.54$) was observed. The inverse relationship was also confirmed at the protein level upon PMA plus ionomycin stimulation (**Figure 1E**) or αCD3/CD28 stimulation of FACS-sorted Treg (**Figure 1F**). As compared to $_{eff}$Treg, $_{naïve}$Treg hardly produced IL-17A, but showed an increased production of TNFα. Analysis of conventional T cells further supported the uniquely high production of IL-17A in these $_{eff}$Treg, as the numbers of IL-17A/FOXP3-positive cells in FACS-sorted naïve or memory $CD4^+CD25^-$ T cells were very low (**Figure S1**).

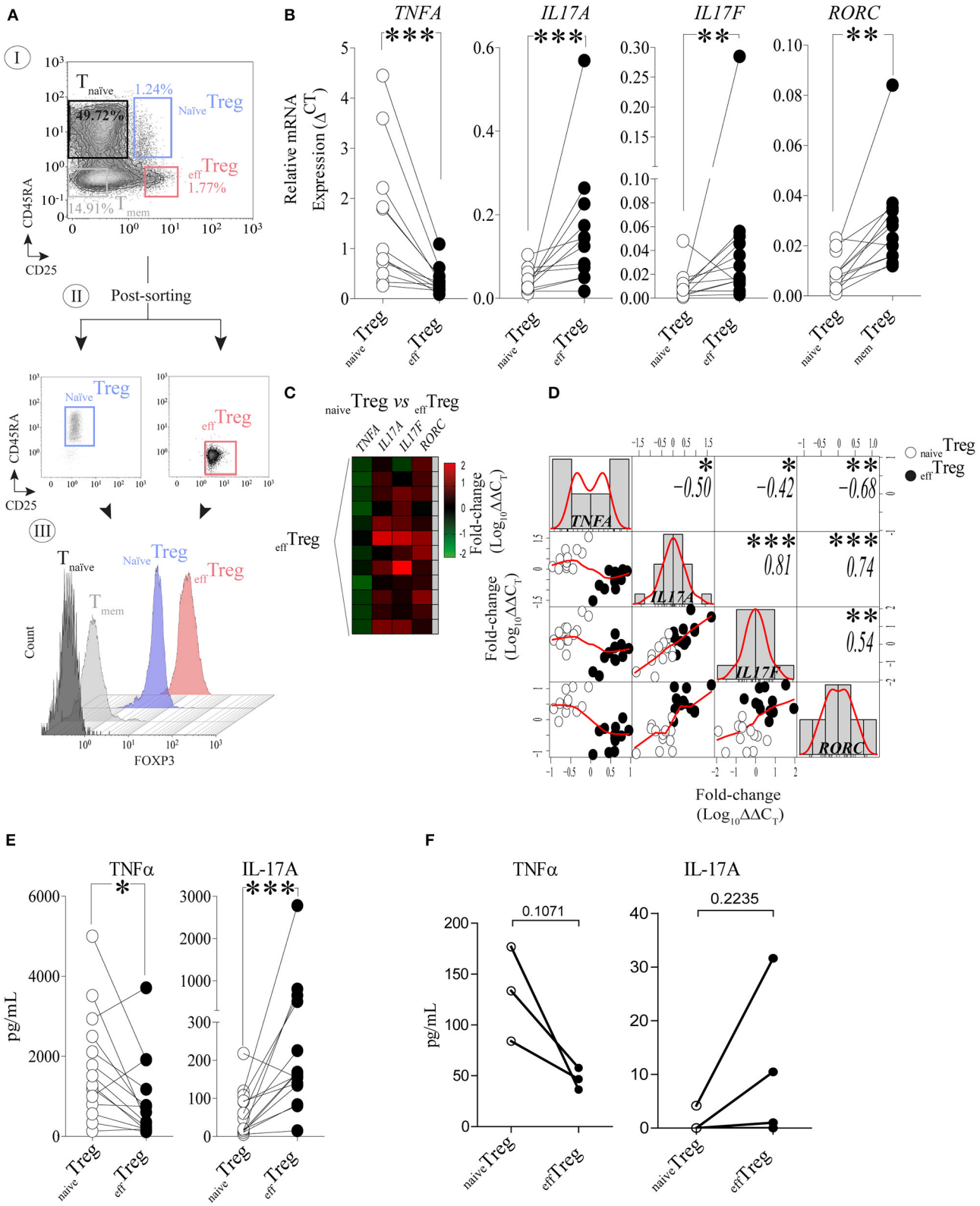

FIGURE 1 | Reciprocal TNFα and IL-17A expression in human $_{naïve}$Treg and $_{eff}$Treg cells. **(A)** An example of the FACS sorting strategy of $_{naïve}$Treg and $_{eff}$Treg based on CD4, CD45RA, and CD25 expression (I. dotplots), post-sorting analysis (II. dotplots) and confirmation of FOXP3 expression in the sorted cell population *(Continued)*

FIGURE 1 | (III. histograms). Conventional $CD4^+CD45RA^-CD25^-$ naïve T cells ($T_{naïve}$), and $CD4^+CD45RA^-CD25^-$ memory T cells (T_{mem}) were sorted and displayed for comparison of FOXP3 expression levels (III). **(B)** RT-qPCR gene expression of *TNFA, IL17A, IL17F,* and *RORC* in $_{naïve}$Treg and $_{eff}$Treg after 20 h of PMA and ionomycin stimulation ($n = 12$). **(C)** Heatmap displaying the fold change of transcripts expression in $_{eff}$Treg within different donors (rows). $_{naïve}$Treg were used as reference to calculate the fold change. **(D)** Multiple correlation matrix depicting the correlation of gene expression in both Treg subsets ($_{naïve}$Treg [open dots] and $_{eff}$Treg [closed dots]). Sample distribution (histogram) is shown, linear regression is also plotted (red lines), whereas *p*-value significance and *r*-values are displayed based on Pearson correlation test. *Y* and *X* axes depict the log10-fold change of *TNFA*, *IL17A*, *IL17F*, and *RORC* expression. Each column represents a gene; in every intersection (rows), we observe the correlation between genes. **(E)** Presence of the cytokines TNFα and IL-17A in culture supernatant after overnight stimulation of Treg subsets using PMA and ionomycin. Cytokines were measured using Luminex ($n = 14$). **(F)** Presence of TNFα and IL-17A in culture supernatants of αCD3/CD28/rhIL-2 activated Treg subsets after 5 days of culture ($n = 3$, mean ± SEM). For statistical analysis, Wilcoxon matched-pairs signed-ranks test **(B,E)**, or two-way ANOVA followed by a Bonferroni *post-hoc* test **(F)** were used. $*p < 0.05$, $**p < 0.01$,$***p < 0.001$, ns, not significant.

TNFα-TNF Receptor-2 Signaling Regulates IL-17A Expression via Ubiquitin-Editing *TNFAIP3*/A20 Protein in Effector $CD4^+$ Regulatory T Cells

Under the stimulation conditions mentioned above, $_{eff}$Treg, but not $_{naïve}$Treg, demonstrated a clear capacity to produce IL-17A; therefore, we focused our further experiments primarily on $_{eff}$Treg. To analyze if TNFα signaling regulates IL-17A expression in $_{eff}$Treg, FACS-sorted $_{eff}$Treg were stimulated with αCD3/CD28-beads plus rhIL-2 and supplemented with either soluble recombinant human (rh)TNFα or the anti-TNFα agent etanercept (ETN, here referred to as anti-TNF), which is a fusion protein of TNF receptor 2 and IgG1 Fc, which neutralizes TNFα and prevents TNFα signaling. Supplementation of rhTNFα as compared to supplementation of anti-TNF, resulted in a significant reduction of IL-17A expressing FOXP3+ $_{eff}$Treg (*p 3.19e-07*) (**Figure 2A**). At the transcriptional level, we demonstrated that supplementation of rhTNFα suppressed IL-*17A*, IL-*17F,* and *RORC* gene expression in $_{eff}$Treg (**Figure 2B**). These data support the idea that TNFα signaling controls IL-17A expression in $_{eff}$Treg.

TNFα binding to its receptors (TNFR1 and TNFR2) leads to a cascade of intracellular events that culminate in NFκB translocation to the nucleus and subsequent transcription of NFκB target genes *NFKBIA* (encode Iκβα), *NFKB1* (encode p50), and *NFKB2* (encode p52) (38, 39). Therefore, we analyzed the effect on the expression of NFκB target genes in $_{eff}$Treg after αCD3/CD28 stimulation with and without supplementation of rhTNFα or anti-TNF. Supplementation with rhTNFα led to a significant increase of *NFKBIA and NFKB2* expression, indicating that TNFα signaling promotes the expression of NFκB target genes, an indication of NFκB activation during Treg activation, while anti-TNF suppressed the NFκB pathway (**Figure 2C**). We previously found that TNFα signaling enhanced *TNFAIP3* (tumor necrosis factor-induced protein 3) expression in conventional T cells (20). *TNFAIP3* encodes the ubiquitin-editing enzyme A20, which in turn regulates NFκB activity. Here, we also observed that TNFα signaling regulated *TNFAIP3* expression in $_{eff}$Treg (**Figure 2D**). To demonstrate causality between suppression of *TNFAIP3* and enhanced expression of IL-17A, we carried out a small interfering RNA assay (siRNA) to inhibit *TNFAIP3* transcription. siRNA-mediated *TNFAIP3* inhibition of $_{eff}$Treg, similar to TNFα signaling inhibition by anti-TNF treatment, led to enhanced *IL-17A* gene expression (**Figures 2E,F**).

As TNFα can bind to both TNFR1 and TNFR2, we measured the expression of these receptors on freshly isolated $_{eff}$Treg and demonstrated that they expressed TNFR2, but TNFR1 was hardly detected (**Figure 2G**). The latter agrees with previous studies (20, 40) and suggests that TNFα-mediated regulation of IL-17A expression in $_{eff}$Treg might be primarily mediated via the TNFR2. To examine this, αCD3/CD28-stimulated $_{eff}$Treg were cultured in the absence and presence of a specific TNFR2 agonist for 5 days. TNFR2 agonist stimulation led to a reduction in the percentages of IL-17A expressing FOXP3+ cells (**Figure 2H**). This indicates that IL-17A expression in $_{eff}$Treg subsets is regulated via TNFα-TNFR2 signaling. Together, these data suggest that TNFα signaling via TNFR2 promotes the expression of the anti-inflammatory mediator *TNFAIP3*/A20, which seems to prevent IL-17A expression in regulatory T cells, as ablation of TNFα signaling suppresses *TNFAIP3*/A20 and results in increased IL-17A expression in human Treg.

TNFα Suppresses T-Cell Receptor and Janus Kinase Protein Activity and Regulates IL-17A Expression in Effector Regulatory T Cells

TNFAIP3/A20 has been demonstrated to regulate critical proteins involved in TCR (23), TNFα (41), IL-17R (21), and Wnt signaling (20, 42). Recently, we demonstrated that the prevention of TNFα signaling in conventional $CD4^+$ memory T cells leads to inhibition of *TNFAIP3*/A20 expression, which subsequently leads to enhanced IL-17A expression (20). *TNFAIP3*/A20 has been shown to regulate kinase activity (21, 23). To better understand kinase regulation by TNFα signaling in $_{eff}$Treg, we here profiled the activity of ∼300 kinases in FACS-sorted $_{eff}$Treg following stimulation with αCD3/CD28 beads in the absence or presence of anti-TNF or rhTNFα. Subsequently, we analyzed the threonine/serine and tyrosine kinase activity using a multiplex human kinase activity array. This kinome array employs ∼300 peptide substrates with known phosphorylation sites and provides a reliable and high-throughput kinase profiling tool for further pathway elucidation (see *Materials and Methods*) (43). We found 30 unique and differentially activated kinases following anti-TNF vs. rhTNFα supplementation comparison (**Figure S2**). For the kinase activity profiling, we focused on

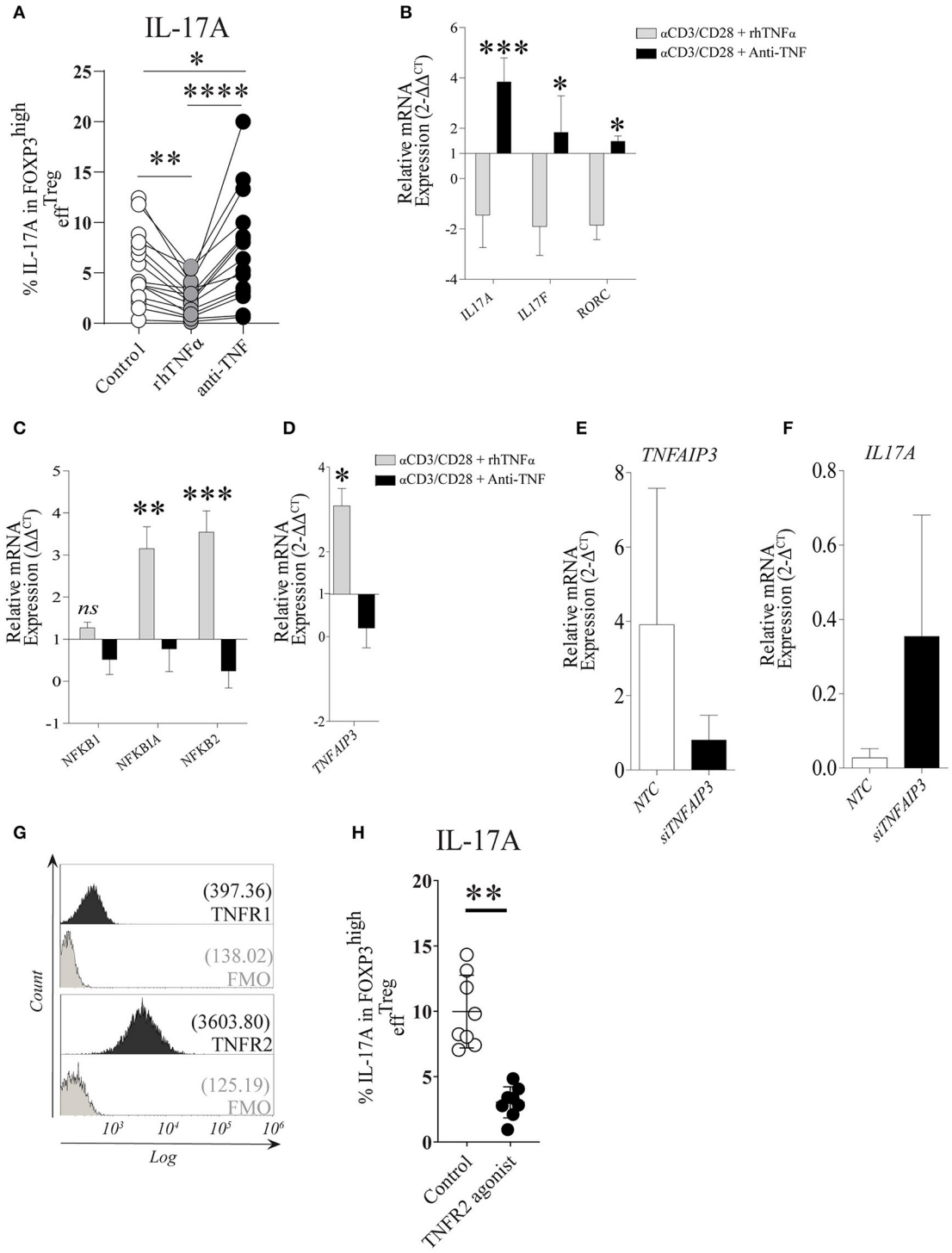

FIGURE 2 | TNFα-TNFR2 signaling reduces IL-17A expression in activated $_{eff}$Treg, conceivably via the anti-inflammatory regulator *TNFAIP3/A20*. **(A)** Flow cytometry of intracellular IL-17A expression in FOXP3^{high} $_{eff}$Treg that were stimulated with αCD3/CD28/rhIL-2 for 5 days in the absence or presence of rhTNFα or anti-TNF ($n = 15$). **(B)** RT-qPCR gene expression of *IL-17A, IL-17F,* and *RORC,* **(C)** NFκB target genes *NFKB1, NFKB1A, NFKB2* ($n = 5$), and **(D)** *TNFAIP3* ($n = 8$) at day 4 of culture. **(E,F)** *TNFAIP3* and *IL-17A* gene expression of non-targeting-gene control (NTC) and *siTNFAIP3* $_{eff}$Treg after 6 days under αCD3/CD28/rhIL-2 stimulation ($n = 3$). **(G)** Histogram depicting the expression of TNFR1 and TNFR2 on $_{eff}$Treg directly after FACS sorting ($n = 9$). **(H)** Flow cytometry of IL-17A expression in FOXP3^{high} $_{eff}$Treg that were stimulated with αCD3/CD28 beads plus rhIL-2 with or without TNFR2 agonist for 5 days ($n = 9$). All data are shown as mean ± SEM. For statistical analysis, a Friedman test followed by Dunn's multiple comparison test **(A)**, a two-way ANOVA followed by a Bonferroni posttest **(B,C)**, and a Wilcoxon matched-pairs signed-rank test **(D,G,H)** were used. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$, ****$p < 0.0001$, ns, not significant.

the two most extreme states of TNF pathway signaling and addressed the differential kinase activity profile following $_{eff}$Treg activation following TNF vs. anti-TNF supplementation. The obtained kinome data were visualized using a volcano plot that shows the fold change of kinase activity and the associated level of significance (*p*-values) (**Figure 3A**, left panel; raw data **Table S1**). We found that inhibition of TNFα signaling, as compared to the supplementation of rhTNFα, in activated $_{eff}$Treg significantly promoted the activity of multiple kinases (red symbols indicate $p < 0.05$). The ranked log2-fold changes of kinase activity are shown in the right panel of **Figure 3A**. Notably, several of the kinases were related to TCR signaling [CD3ζ (CD247), CD3ε, ZAP70, and Lck] (44). Also, cell cycle regulating (CALM, CD28, GSK3B, MAPK3, PGR, and JAK3) (45, 46) and apoptosis (ANXA2, Annexin V) (47)-related kinases were induced.

To obtain a more comprehensive understanding of the kinase network and cellular pathways regulated by neutralization of TNFα, the kinases that were significantly activated following anti-TNF mAb treatment were analyzed using STRING (*Search Tool for the Retrieval of Interacting Genes/Proteins*). STRING is a web-based biological resource (https://string-db.org) of known and predicted protein–protein interactions enabling prediction of the functional protein association network of a group of given proteins by estimating the likelihood of meaningful biological interactions (48). In our analysis, we used the highest confidence interaction score (0.900) to associate all kinases that were significantly activated following anti-TNF treatment as listed in the right panel of **Figure 3A**. STRING association analysis demonstrated that inhibition of TNFα signaling in activated $_{eff}$Treg involved prominent immune signaling pathways such as the PKC, p38-MAPK, and JAK pathways, which were all linked to TCR signaling [CD3ζ (CD247) and CD3ε] (**Figure 3B**). Previously, these pathways were shown to be associated with the induction of IL-17A expression (49–52).

To validate if the predicted pathways were indeed involved in rhTNFα-induced suppression of IL-17A expression in $_{eff}$Treg, FACS-sorted $_{eff}$Treg were activated in the presence or absence of anti-TNF and specific kinase inhibitors of JAK/STAT (Tofacitinib), PKC (AEB071, Sotrastaurin), or p38 MAPK (UR13870). For the inhibition of TCR signaling, an Lck inhibitor (A420983) was applied. We demonstrated that suppression of JAK, and Lck kinases, but not PKC and p38, prevented the expression of IL-17A expression in $_{eff}$Treg that were activated under TNFα signaling inhibiting or not (**Figure 3C**). In fact, suppression of JAK and Lck inhibited the expression of IL-17 similar to the TNF supplementation condition. The inhibitors tested did not affect FOXP3 expression (**Figure S3**).

Next, we performed a functional ontology enrichment analysis of the most significant biological process networks, processes, and diseases by submitting the kinase data that we identified in activated $_{eff}$Treg following supplementation vs. inhibition of TNFα to MetaCore™ database analysis. Significant enriched MetaCore™ GO process networks involved immune response-TCR signaling, cell cycle regulation, and lymphocyte proliferation (**Figure 4A**). The most significantly enriched MetaCore™ Go processes based on the submitted kinases were kinase signaling pathways via transmembrane receptor protein tyrosine, signal transduction processes, and tyrosine phosphorylation and modification (**Figure 4B**). Furthermore, there was an enrichment of cell communication and cell development processes. MetaCore™ Go diseases indicated a strong enrichment of autoimmune disease, next to other pathological conditions ranging from the nervous system, nutritional, and metabolic disorders (**Figure 4C**). Together, these data demonstrated that CD3 and CD28 activation of $_{eff}$Treg in the absence of TNF-signaling by anti-TNF treatment promotes tyrosine kinase activity of relevant TCR-associated signaling pathways.

DISCUSSION

Human Treg can express the pro-inflammatory cytokine IL-17A under specific conditions; a phenomenon referred to as Treg plasticity (5, 8). The molecular mechanisms regulating this phenomenon are not well-understood. In our current work, we demonstrate that TNFα signaling regulates IL-17A expression in $_{eff}$Treg by controlling a kinase activity network that includes TCR linked kinases and other prominent immune signaling kinase pathways such as the JAK pathway. Also, TNFα-mediated regulation of the anti-inflammatory mediator *TNFAIP3*/A20 appeared crucial to control IL-17A expression by $_{eff}$Treg. TNFR2 is the main receptor for TNFα signaling in Treg. TNFR2 stimulation has been demonstrated to support Treg stability (18, 19, 25, 53), whereas the effect of TNF signaling on the stability of Treg is ambiguous (54, 55). Here, we show that TNFR2 is highly expressed on human $_{eff}$Treg, and TNF-TNFR2 signaling in $_{eff}$Treg acts as a negative regulator of IL-17A expression by controlling TCR and JAK signaling.

STRING association analysis revealed that inhibition of TNFα signaling is associated with increased TCR associated signaling of CD3ζ, CD3ε, ZAP70, and Lck, indicating that TNFα signaling in $_{eff}$Treg functions as a rheostat of TCR signal transmission. Although information of TNFα stimulation on the TCR signaling in Treg is lacking, it has been shown in CD4+ T cells of both mice and man that TNFα stimulation results in specific down-regulation of TCRζ expression and impaired TCR/CD3 signaling, including phosphorylation of the TCRζ, CD3ε, ZAP-70 tyrosine kinase, and linker for activation of T cells (LAT) (56). TCR signaling is essential for both effector and regulatory T cells (57). Treg have a more extensive TCR repertoire than effector T cells, and TCR signaling is crucial for proper Treg function (58–61). Signaling via the T cell antigen receptor of Treg is critical for FOXP3 expression and their suppressive activity. Mutations resulting in signaling-deficient TCRζ chains led to increased Treg numbers with higher suppressive activity (62–64). Reduced TCR signaling will alleviate downstream signaling and favor Treg cell lineage commitment. TNFα signaling, as we demonstrate here, seems to safeguard TCR-related kinase activity in $_{eff}$Treg and stabilize Treg function as illustrated by preventing IL-17A expression. Note that anti-TNF had a mild effect on the induction of IL-17A expression in $_{eff}$Treg, which is in contrast to its clear induction of IL-17A in conventional memory T cells (20). This phenomenon may be caused by the poor intrinsic

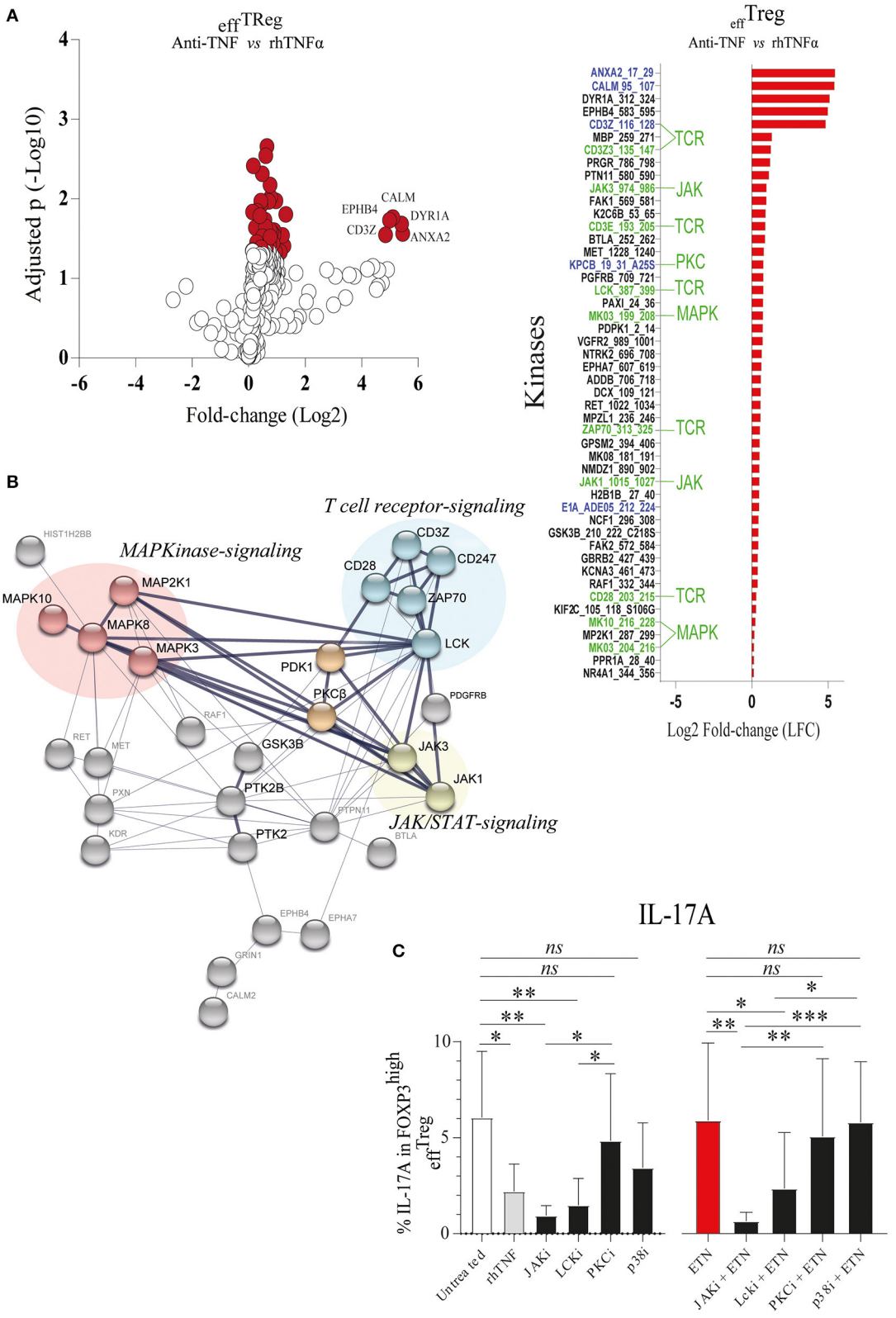

FIGURE 3 | TNFα signaling in $_{eff}$Treg suppresses TCR and JAK kinase activity, leading to regulation of IL-17A expression. $_{eff}$Treg were stimulated with αCD3/CD28 beads and rh-IL-2 in the presence of rhTNFα or anti-TNF. On day 4, phosphoserine/threonine kinase (STK) and phosphotyrosine kinase (PTK) activity of cells were

(Continued)

FIGURE 3 | Analyzed using a kinome activity array. **(A)** Left panel: Volcano plot showing the fold change in kinase activity and adjusted *p*-values (red symbols, $p < 0.05$; $n = 4$) in STK and PTK kinase activity. Right panel: Fold change in the kinases identified by comparing anti-TNF with rhTNFα conditions. Of note, TNFα was used as reference to calculate the fold change. Green texts indicate unique kinases that show increased activity upon comparison of anti-TNF to rhTNFα conditions; Blue texts represent kinases with enhanced activity upon comparison of comparing anti-TNF to the control (αCD3/CD28 stimulated without rhTNF or anti-TNF). **(B)** Cumulative STRING© protein network analysis based on the identified kinases listed in **(A)**. **(C)** Flow cytometry of intracellular IL-17A expression in $FOXP3^{high}$ $_{eff}$Treg. Pathway inhibition validation assays applying small chemical molecules in the stimulation assay as described above (mean ± SEM, $n = 7$). JAKi, JAK inhibitor (tofacitinib); Lcki, Lck inhibitor (A420983); PKCi, PKC inhibitor (AEB071); and p38i, p38MAPK inhibitor (UR13870). ANOVA Dunnett's testing **(A)** and Friedman test followed by Dunn's multiple comparisons test **(C)** were used. $*p < 0.05$, $**p < 0.001$, $***p$ 0.0001, ns, not significant.

capacity of $_{eff}$Treg to produce TNFα *in vitro*. In fact, highly pure FACS-sorted $_{eff}$Treg barely produced TNFα (41.35 pg/ml ± 6.75), whereas memory conventional T cells produced significantly higher levels (335.7 pg/ml ± 65.33, $n = 4$) (data not shown).

Next to TCR-derived signals, Treg integrates inputs from cytokine, chemotactic, and metabolic cues to fulfill their function optimally. Proximal cytokine signaling often takes place via JAK-STAT signaling (65). *IL-17A* gene transcription is associated with JAK-STAT3 signaling (66). Inhibition of TNFα signaling using anti-TNF inhibitor ETN was associated with increased JAK1 and JAK3 kinase activity in αCD3/CD28 stimulated $_{eff}$Treg. Inhibition of JAK1 and JAK3 kinase activity by the clinically applied JAK inhibitor tofacitinib prevented IL-17A expression in anti-TNF-treated $_{eff}$Treg, suggesting that TNFα signaling is involved in driving JAK/STAT signaling. Although TNFα is not a prototypic JAK/STAT activating cytokine, the anti-inflammatory molecule A20 (encoded by *TNFAIP3*) that is a downstream target of TNFα signaling acts as a regulator of STAT (67, 68). The absence of A20 in myeloid cells resulted in enhanced STAT1-dependent inflammation (68). This relationship needs to be confirmed in $_{eff}$Treg.

Although anti-TNF therapy is improving the life quality of many patients with chronic inflammatory diseases, 10–20% of patients do not respond to the treatment while 0.6–5% of patients treated with TNF inhibitors reveal paradoxical immune-mediated inflammatory side effects (36, 37). Although the mechanism of the latter phenomenon is not fully understood, it might be of interest to consider an additional JAK inhibitor treatment such as tofacitinib or other JAK inhibitors to prevent the putative IL-17A expression by Treg. Also, regarding Treg-based immune therapy in transplantation or autoimmunity, the clinical design has started to consider strategies to minimize the risks of Treg plasticity (69) at the time of ex vivo production and following *in vivo* administration (70, 71). Our results suggests that TNFα-TNFR2 signaling or inhibition of JAK signaling might favor Treg stability. Along with this line of reasoning, it has been demonstrated that JAK inhibition (72) as well as TNFR2 stimulation (18, 19) support human Treg function and prevent Treg plasticity.

In conclusion, we demonstrated an inverse production of TNFα and IL-17A between human naïve and effector Treg cells. Supplementation of rhTNFα led to a down-regulation in the frequency of IL-17A-producing $_{eff}$Treg, mainly via the activation of NFkB pathway as well as the up-regulation of *TNFAIP3/A20* expression. TNFR2 receptor seems to play a crucial role since we hardly detected any expression of TNFR1 on $_{eff}$Treg and treatment of $_{eff}$Treg with TNFR2 specific agonist resulted in a similar inhibition of IL-17A production. Accordingly, inhibition of TNFα signaling using the clinically applied anti-TNF inhibitor ETN led to decreased *TNFAIP3* and increased *IL-17A* expression, a phenomenon similar to what is observed in human conventional memory CD4+ T cells. Kinome activity screening of αCD3/CD28 stimulated $_{eff}$Treg revealed that anti-TNF led to an increase in kinase activity of multiple kinases including CD3ζ (CD247) and LcK. A functional ontology enrichment analysis indicated that these kinases were highly associated with different immune response signaling pathways including TCR-, JAK-mediated pathways. We propose that these findings might be relevant for optimizing anti-TNF-based therapy and may aid in preventing Treg plasticity in case of Treg-based cell therapy.

MATERIALS AND METHODS

Study Approval

The protocols of this study were performed in agreement with the Declaration of Helsinki and in accordance with the Radboud university medical center (Radboudumc) in Nijmegen, the Netherlands.

Subjects

Blood buffy coats from voluntary donors were purchased from the Sanquin Blood Bank, Nijmegen, the Netherlands. The volunteers gave written informed consent.

Regulatory T Cell Isolation

$CD4^+$ T cells were isolated using RosetteSep™ Human $CD4^+$ T cell enrichment cocktail 25–50 µl of cocktail/ml of blood (StemCell Technologies, Vancouver, Canada) according to the instructions of the supplier. To sort $CD4^+CD25^+CD45RA^+$ ($_{naïve}$Treg) and $CD4^+CD25^{high}CD45RA^-$ ($_{eff}$Treg), the purified $CD4^+$ cells were washed and stained with anti-CD25-BV510 (*M-A251,* BD, New Jersey, USA), anti-$CD45RA^-$ PE (*4KB5,* Dako, Brüsseler Straße, Germany), CD4-PE-Cy5.5 (*13B8.2,* Beckman-Coulter, California, United States), and FACS-sorted on a FACSAria™ III machine (BD Biosciences, New Jersey, United States). The gating strategy during FACS sorting, post-sorting purity analysis, and confirmation of FOXP3 expression in freshly sorted cell subsets are described in **Figure 1A**. The purity of the sorted cell populations was 95.3 ± 4.1% (mean ± SD).

Cell Culture

RPMI-1640 Dutch modified (Gibco, Massachusetts, United States) culture medium, containing sodium bicarbonate

FIGURE 4 | Enrichment analysis of the kinome array data. Functional ontology enrichment analysis using the MetaCore™ database reveals **(A)** the distinct biological networks, **(B)** the different biological processes, and **(C)** various diseases related to kinases identified in **Figure 3**. The probability of a random intersection between the set of kinases with ontology entities was estimated with the "*p*" value of the hypergeometric intersection. A lower "*p*" value means higher relevance of the entity to the dataset, which appears in higher rating for the entity.

and 20 mM HEPES, supplemented with penicillin/streptomycin (100 U/ml), sodium pyruvate (1 mM), glutamine/glutamax, and 10% human pooled serum (HPS, Radboudumc), was used in all experiments. After cell isolation, 2.5×10^4 cells/well were cultured in 96-well U-bottom plates and stimulated with Dynabeads® Human T-Activator CD3/CD28 (αCD3/CD28 beads, 1:5 of bead:cell ratio) (Gibco, Massachusetts, United States) in the presence of recombinant human (rh) IL-2 (rhIL-2, 100 U/ml) (Proleukin Prometheus Laboratories, California, United States). In some conditions, cultures were

supplemented with rhTNFα (50 ng/ml, R&D, Minnesota, United States), or TNFα inhibitors etanercept (5 μg/ml; ETN—Enbrel, Pfizer, New York, United States), or TNFR2 agonist (2.5 μg/ml, Clone *MR2-1*, Hycult Biotech, Uden, the Netherlands). To examine the effect of a pharmaceutical inhibitor, tofacitinib (0.112 μM, Pfizer, New York, United States), PKC inhibitor Sotrastaurin (1 μM), Lck inhibitor A420983 (1 μM), or p38α/β kinase inhibitor UR13870 (10 μM) was pre-incubated with the FACS-sorted cells for 30 min before the addition of any stimulus. In some cases, cells were stimulated with PMA (12.5 ng/ml) and ionomycin (500 ng/ml) for 20 h.

Flow Cytometry

Flow cytometry was performed using a 10-color Navios Flow cytometer (Beckman Coulter, California, United States), which is equipped with blue (488 nm), red (638 nm), and violet (405 nm) lasers. For surface staining, the following antibodies were used: anti-CD3-ECD (*UCHT1*), anti-CD45RA-ECD (*2H4LDH11LDB9*), anti-CD45-KO (*J33*), anti-CD4-PE-Cy5.5 (*13B8.2)*, and anti-CD8-APC-AF700 (*B9.11*) (all from Beckman-Coulter); anti-TNFR1-AF488 (*16803*, R&D); and anti-TNFR2-APC (*22235*, R&D). For intracellular staining, the following antibodies were used: anti-IFNγ-PE-Cy7 (*4S.B3*) and anti-IL-17A-AF-660 (*eBio64DEC17*) (eBioscience, California, United States). Unstained (Fluorescence Minus One, FMO) samples were also measured to help set the gates during data analysis. To evaluate cytokine production, we challenged the cultured Treg subsets for another 4 h with PMA (12.5 ng/ml), ionomycin (500 ng/ml), and Brefeldin A (5 μg/ml) (Sigma-Aldrich, Missouri, United States) before performing the FACS staining process. Briefly, cells were stained with the fixable viability dye-eFluo 780 (FVD, eBioscience) for 30 min at 4°C, following with surface mAb staining, cell fixation, and permeabilization by using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience) and intracellular mAb staining. For flow cytometry data analysis, Kaluza1.5 software (Beckman Coulter) was used.

Small Interfering RNA Transfection

For small interfering RNA (siRNA) knockdown of *TNFAIP3*, Accell SMARTpool siRNA (Dharmacon, Colorado, United States) was used according to the manufacturer's instructions. Briefly, 1×10^5 $_{eff}$Treg cells per well were stimulated with αCD3/CD28 beads (1:5 of bead:cell ratio) in Accell Delivery Medium (Dharmacon) supplemented with rhIL-2 (100 U/ml) and incubated with 1 mmol cyclophilin B siRNA (positive control), or 1 mmol non-targeting control siRNA, or 1 mmol *TNFAIP3* siRNA for 120 h (for siRNA sequences, see **Table S2**). Quantitative real-time PCR (RT-qPCR) was performed to confirm the knockdown of the target gene expression.

RT-qPCR

Total RNA was extracted by using the RNeasy Plus Micro Kit (Qiagen) followed by cDNA synthesis using the SuperScript III First-Strand Synthesis System and Oligo(dT)20 primer (Thermo Fisher Scientific, Massachusetts, United States). TaqMan gene expression assays were purchased from Thermo Fisher Scientific (**Table S3**). RT-PCR was acquired in a 7500 Real-Time PCR System (Applied Biosystems). RT-qPCR cycle values (C_T) obtained for specific mRNA expression in each sample were normalized to the C_T values of human *HPRT1* (endogenous control), resulting in ΔC_T values (log ratio of the gene concentrations) that were used to calculate the relative gene expression.

$$\Delta C_T = Mean\ CT - Housekeeping\ gene\ Mean\ CT$$

Then, we performed an exponential conversion of ΔC_T, namely, $2^{-\Delta CT}$ using the following formula:

$$2\hat{}(exponential) - \Delta CT$$

$2^{-\Delta CT}$ representing the relative gene expression was used in **Figures 1B,E,F**.

$_{eff}$Treg stimulated in the absence of anti-TNF or rhTNFα were used as a baseline to calculate the relative gene expression in fold change ($\Delta\Delta C_T$) for $_{eff}$Treg stimulated in the presence of rhTNFα *vs.* ETN treatment.

$$\Delta\Delta C_T = Mean\ \Delta CT - Mean\ \Delta CT\ reference\ sample\ (control)$$

Subsequently, we performed an exponential conversion of $\Delta\Delta C_T$, namely, $2^{-\Delta\Delta CT}$ using the following formula:

$$2^{-\Delta\Delta CT} = 2\hat{}(exponential) - \Delta\Delta CT$$

$2^{-\Delta\Delta CT}$ representing the relative gene expression in fold change was employed for **Figures 2B–D**. In **Figures 1C,D**, log10 $\Delta\Delta C_T$ was employed. The Relative Quantification app (Thermo Fisher Scientific cloud) was used for data analysis.

Measurement of Cytokines Secretion

The cell culture supernatants were analyzed for the presence of IL-17A, IFNγ, and TNFα using Bio-Plex Pro Human Th17 Cytokine Assays (Bio-Rad, California, United States) according to the manufacturer's instruction. The cytokine concentrations were measured using a Luminex^{100} machine (Luminex Corp., Texas, United States). The lowest limit of detection was <1.870 pg/ml for IL-17A, <2.411 pg/ml for IFNγ, and <2.231 pg/ml for TNFα.

Protein Kinase Chip Assay

After sorting and stimulations of cells, samples were frozen for further analysis. The protein isolation was performed according to the manufacturer's instruction (P1160, PamGene International B.V., 's-Hertogenbosch, the Netherlands). Kinase activity was measured with PamGene's Protein Tyrosine Kinase (PTK) PamChip (Cat. number 86402) and Serine Threonine kinase (STK) PamChip (Cat. number 87102). Each PTK PamChip array contains 196 peptides immobilized on a porous membrane, whereas each STK PamChip array contains 144 peptides (see the full list of peptides at www.pamgene.com). The peptide sequences (13 amino acids long) harbor phosphorylation sites, defined based on literature or derived from computational

predictions and are correlated with one or multiple upstream kinases. A fluorescently labeled anti-phospho-Tyr antibody (PY20) is used to detect the phosphorylation activity of tyrosine kinases present in the sample. For the STK assay, an antibody mix is used to detect the phosphorylated Ser/Thr, and the 2nd FITC-conjugated antibody is used in a detection mix to quantify the phosphorylation signal. BioNavigator software 6.3 (PamGene) was used to determine signal intensities, peptide quality control (QC) and preselection (phosphorylation kinetics, or increase in signal over time, in 25% of the arrays analyzed), Log 2 transformation, ANOVA-Dunnett's testing, and data visualization. Mapping and pathway elucidation analysis were performed using METACORE^{TM} (Clarivate Analytics, PA, USA) and STRING (73). As described by the GeneGo manufacturer's report, the analysis consists in matching the protein IDs of possible targets for the "common," "similar," and "unique" sets with protein IDs in functional ontologies in MetaCore (73). The lower *p*-value means a higher relevance of the entity to the dataset, which shows a higher rating for the entity.

Statistics

Statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA) and R. For experiments with more than two groups of matched samples, we used non-parametric Friedman test followed by Dunn's Multiple Comparison Test, whereas for experiments with only two groups of matched samples, we employed non-parametric Wilcoxon matched-pairs signed-rank test.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Radboud university medical center (Radboudumc) in Nijmegen, the Netherlands. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PU, IJ, and HK designed the research. PU, OF, XH, HT, and BH performed the experiments. PU, OF, IJ, BH, XH, RS, and HK analyzed the data. PU, XH, IJ, and HK prepared and wrote the final manuscript. All the authors reviewed the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.03047/full#supplementary-material

Figure S1 | Expression of IL-17A in Conventional and regulatory T cells. Sorted naïve T cells ($CD4^+CD45RA^+CD25^-$), memory T cells ($CD4+CD45RA^-CD25^-$), naïve Treg ($CD4^+CD45RA^+CD25^{low}$) and effector Treg ($CD4+CD45RA^-CD25^{high}$) were stimulated with anti-CD3/CD28 bead plus rhIL-2. Cells were harvested on day 5 and intracellular FOXP3 and IL-17A expression were detected by FACS staining.

Figure S2 | Kinome data analysis. Kinase activity of $_{eff}$Treg following αCD3/CD28 bead plus rhIL-2 activation in the absence or presence of anti-TNF or rhTNFα. Significant changes of kinase activities are presented in a Venn diagram (**A**, left panel) and a bar plot that ranked based on the log2 fold-change of kinase activities. **(B,C)** Bar graphs showing significant changes of kinase activity between anti-TNF and αCD3/CD28 control **(B)** or rhTNF and αCD3/CD28 control **(C)**.

Figure S3 | The Janus kinase, Lck, PKC and p38 MAPK inhibitors do not affect FOXP3 expression in $_{eff}$Treg. $_{eff}$Treg were stimulated with αCD3/CD28 beads in the presence or absence of rhTNFα or anti-TNF or small chemical molecules such as JAK inhibitor (tofacitinib), Lck inhibitor (A420983), PKC inhibitor (AEB071) and p38MAPK inhibitor (UR13870) for 5 days. Flow cytometry analysis of intracellular FOXP3 expression ($n = 5$). Data are shown as mean ± SEM.

Table S1 | Kinome Log 2-transformed dataset.

Table S2 | Target genes used for siRNA interference.

Table S3 | Primers used for RT-qPCR.

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REVIEW
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Molecular Mechanisms Controlling Foxp3 Expression in Health and Autoimmunity: From Epigenetic to Post-translational Regulation

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The discovery of the transcription factor Forkhead box-p3 (Foxp3) has shed fundamental insights into the understanding of the molecular determinants leading to generation and maintenance of T regulatory (Treg) cells, a cell population with a key immunoregulatory role. Work over the past few years has shown that fine-tuned transcriptional and epigenetic events are required to ensure stable expression of Foxp3 in Treg cells. The equilibrium between phenotypic plasticity and stability of Treg cells is controlled at the molecular level by networks of transcription factors that bind regulatory sequences, such as enhancers and promoters, to regulate Foxp3 expression. Recent reports have suggested that specific modifications of DNA and histones are required for the establishment of the chromatin structure in conventional $CD4^+$ T (Tconv) cells for their future differentiation into the Treg cell lineage. In this review, we discuss the molecular events that control Foxp3 gene expression and address the associated alterations observed in human diseases. Also, we explore how Foxp3 influences the gene expression programs in Treg cells and how unique properties of Treg cell subsets are defined by other transcription factors.

Keywords: Foxp3, Treg cells, epigenetic regulation, Foxp3 stability, autoimmunity

INTRODUCTION

The key evidence of Forkhead box-p3 (Foxp3) as regulatory T (Treg) cell lineage-specific transcription factor is that its gene mutations lead to autoimmune disease in both mice and humans (1, 2). Foxp3 expression is used for identification of Treg cells [as $Foxp3^+$ T cells in mice (1), $Foxp3^{high}CD45RA^-$ or $Foxp3^+CD127^-CD25^{high}$ T cells in humans (3–5)], and the study of Foxp3 modulation during immune responses is crucial to understand Treg cell homeostasis and function (6). Foxp3 transcription is induced by T cell receptor (TCR) signaling and temporally persistent TCR signals activate Foxp3 transcription in self-reactive thymocytes (7, 8). Upon its expression, an autoregulatory transcriptional circuit stabilizes Foxp3 gene expression to consolidate Treg cell

differentiation and activate the suppressive function (9). However, upon T cell activation, the induction of Foxp3 could represent a negative regulator of immune response (6). While Foxp3 can drive Treg cell development and function by establishing the required cell program, gene expression analysis of $Foxp3^+$ and $Foxp3^-$ T cells suggests that many Treg cell-specific genes are independent of Foxp3, thus changing the paradigm of Foxp3 as the only factor required for the establishment of Treg cell phenotype (10, 11). Accordingly, Foxp3 expression alone is not sufficient to convert non-Treg into Treg cells with a classical Treg-type gene signature/function. The use of chromatin-immunoprecipitation (ChIP), combined with expression array analysis, allowed the identification of several DNA sequences that are directly bound by Foxp3, which can act as transcriptional activators of some genes and repressor of others (12, 13). However, how Foxp3 controls gene expression in relation to Treg cell function is not yet fully understood. In the breach of immunological tolerance and in autoimmunity, changes in the microenvironmental cues perturb the transcriptional and epigenetic regulation of Foxp3, resulting into an impaired Treg cell generation and suppressive function (14, 15). Several studies demonstrated that, during inflammation, Treg cells may lose their phenotypic properties and be converted into effector T cells secondarily to the alteration of Foxp3 expression and stability (16–18). Thus, understanding the regulation of the mechanisms that govern Treg cell differentiation and function and whether/how this regulation may be disrupted in human autoimmune disease are of pivotal importance.

In this review, we summarize the molecular mechanisms controlling the epigenetic, transcriptional, translational, and post-translational regulation of Foxp3 in health and autoimmunity.

TREG CELL FATE DETERMINATION AND STABILITY

Thymus derived Treg (tTreg) cells, which differentiate intrathymically, require high-affinity or high-avidity TCR interactions with *self*-peptides/major histocompatibility complex class II (MHC II) molecules presented by either thymic epithelial cells or dendritic cells (DCs) (1, 19–22). Treg cells can also develop extrathymically by conversion of mature $CD4^+$ T conventional (Tconv) cells into peripherally induced Treg (pTreg) cells, under normal homeostatic and inflammatory conditions (23–26). It is now widely recognized that Foxp3 gene regulation is responsible for both tTreg and pTreg cell generation and required for the acquisition of immunosuppressive properties, thus representing the master regulator of Treg cell lineage commitment (1, 19, 27). These Treg cell subsets are heterogeneous in terms of development, functional activity, and phenotype, but are both essential for the maintenance of immune homeostasis (28–30).

tTreg cells develop from CD4 single-positive thymocytes (31) and their Foxp3 expression is induced upon interaction with cortical and medullary thymic cells (32, 33). TCR signal strength and its duration are crucial in determining the generation of CD4 and CD8 T cell subsets during thymic differentiation (34, 35). In particular, synergistic signals downstream the TCR together with cytokine-mediated stimulation are required for the transcription of Foxp3 gene in Treg cell precursors (27, 36, 37). Together with high-affinity TCR signals, also co-stimulation through CD28 is required for tTreg cell development, as shown by the significant reduction of these cells in CD28-deficient mice as well as in mice deficient for B7-1/B7-2, the two ligands of CD28 (38, 39). Lymphocyte-specific protein tyrosine kinase (Lck) binding to the CD28 cytosolic tail is one of the events leading to Treg cell differentiation program in thymocytes (38). Since a TCR with increased *self*-reactivity can also be expressed by a non-Treg cell, other factors are necessary to drive Treg cell lineage commitment. In particular, interleukin (IL)-2 plays a fundamental role in Treg cell homeostasis and differentiation (40), a role discovered through the use of different mutant mice. Rudensky and colleagues observed reduced number of tTreg cells in IL-2-deficient compared to wild-type mice (41). However, there are some discordant studies that found normal numbers of tTreg cells in IL-2-deficient or IL-2 receptor (R)α-deficient mice (41–44), thus indicating that IL-2 may play a role in Treg cell development but may not be strictly required. IL-15, which shares the IL2-Rβ chain with IL-2, may also be involved in tTreg cell generation but, again, conflicting studies do not confirm its absolute requirement (42, 44). Transforming growth factor-β (TGF-β) is also important for the thymic differentiation of Treg cells, with mouse conditional deletion of TGF-β receptor I (TGF-βRI) in the first week of life leading to drastically reduced Treg cell differentiation (45–47). Another study has suggested a possible anti-apoptotic role for TGF-β that would enhance tTreg cell survival and thus contribute to their stability (48).

Both overlapping and distinct signaling pathways drive the generation of pTreg cells stably expressing Foxp3 such as cytokine milieu rich in TGF-β and IL-2 during antigen presentation mediated by certain DCs subsets, antigen concentration, the dose and the duration of TCR stimulation, the costimulatory molecule CD28, and IL-2/IL-2R signaling (26, 49–51). Moreover, TGF-β drives the induction of Foxp3 in pTreg cells from both murine and human Tconv cells; Foxp3 in turn downregulates the small mother against decapentaplegic (Smad)7 protein, thus suppressing the key negative regulator of TGF-β signaling (52).

Treg cell lineage is stable with minimal capacity to de-differentiate and convert into effector T (Teff) cells. Nonetheless, there exist pathological conditions in which $CD25^{low}$ Treg cells with an unstable Foxp3 expression are converted into Tconv cells (16). Using whole-genome methylated DNA immunoprecipitation sequencing, Ohkura and colleagues have observed that Treg cells support a distinct DNA methylation pattern compared to Tconv cells and specific epigenetic mechanisms critically influence Foxp3 stability (53, 54).

Since the maintenance of a stable and functional pool of Treg cells is crucial to ensure proper immune tolerance and homeostasis, it is relevant to deeply understand the epigenetic mechanisms and factors that stabilize Foxp3, on which the balance between tolerance and autoimmunity depends.

EPIGENETIC PROFILE OF THE FOXP3 LOCUS

Increased evidence has recognized that epigenetic modifications occurring in the regulatory regions of Foxp3 locus are key determinants in Treg cell commitment (55–57). Besides the Foxp3 promoter, the three conserved non-coding sequences (CNS) within the locus, i.e., CNS1, CNS2, and CNS3, are also targets of several modifying enzymes and are regulated at different stages of Treg cell development (58). CNS1, situated downstream of the Foxp3 promoter, seems not to be essential for tTreg cell development, but reduced frequency of pTreg cells in gut-associated lymphoid tissue (GALT) and mesenteric lymph nodes (MLN) in Foxp3$^{\Delta CNS1-gfp}$ (CNS1-KO) mice instead indicates the importance of CNS1 region during Foxp3 induction in peripheral CD4^{+} T cells (58). Since GALT and maternal placenta are highly enriched in pTreg cells, CNS1-deleted mice are also characterized by increased mucosal Th2 inflammation and abortion rate (59–61). To further confirm the role of CNS1 in pTreg cell generation, Schuster and colleagues have observed that CNS1 deficiency impairs pTreg cell formation in non-obese diabetic (NOD) mice, and this phenomenon is correlated with more severe insulitis (62). DNA methylation experiments have revealed that both Foxp3 promoter and CNS2 are highly CpG demethylated in tTreg cells, opening the Foxp3 locus in Treg cell precursors and favoring Foxp3 mRNA transcription and lineage stability [**Figure 1**; (56, 58)]. The CNS2 region is highly rich in CpG motifs and indispensable for Treg cell

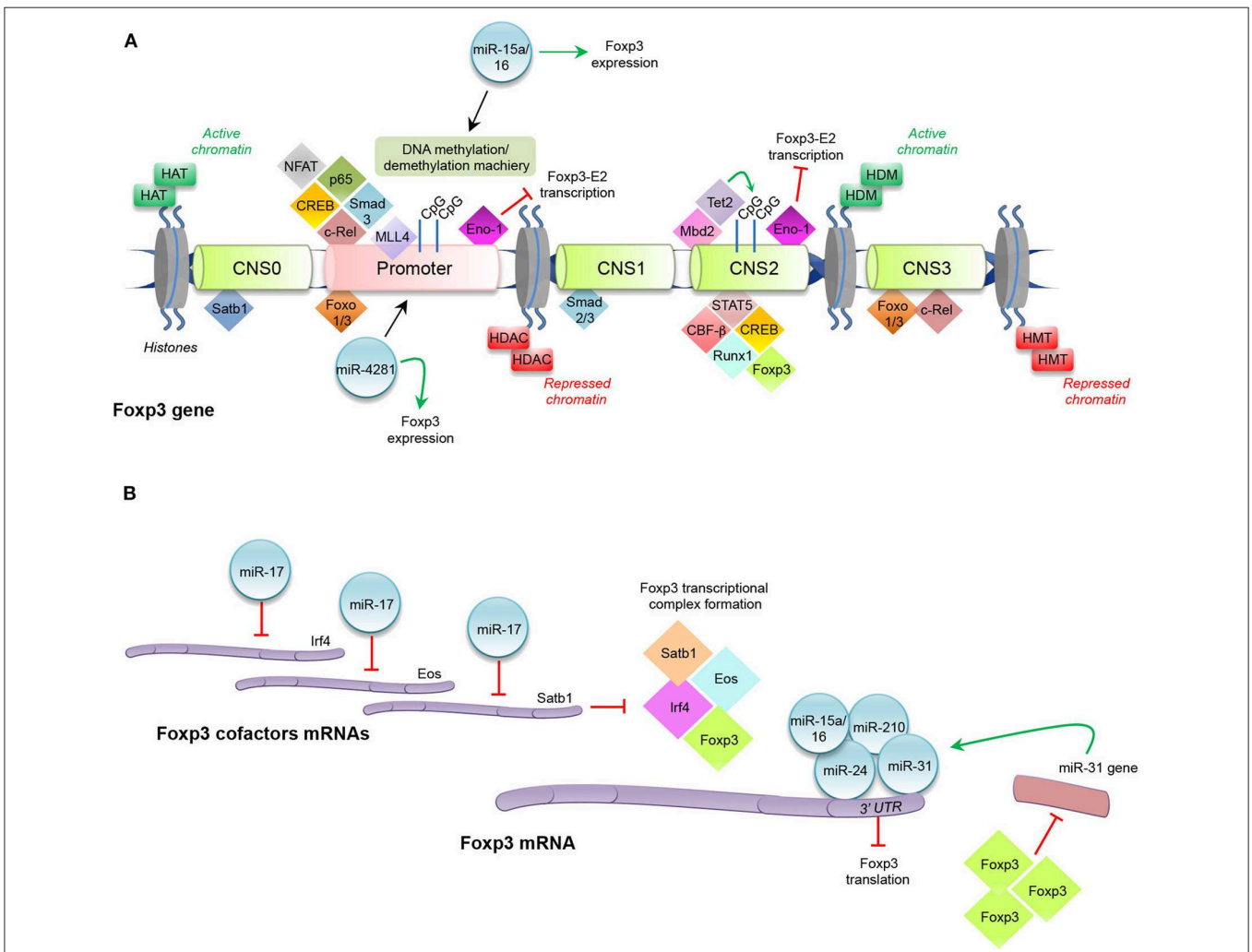

FIGURE 1 | Histone modifications, transcription factors, and miRNAs regulating Foxp3 expression. **(A)** Enzymes catalyzing histone modifications: histone acetyltransferase (HAT), histone deacetylases (HDAC), histone methyltransferase (HMT), and histone demethylase (HDM). Transcription factors binding promoter and conserved non-coding sequences (CNS0, CNS1, CNS2, and CNS3) at Foxp3 locus. Promoter: nuclear factor of activated T-cells (NFAT), p65, cAMP response element binding protein (CREB), mothers against decapentaplegic (Smad)3, c-Rel, mixed lineage leukemia (MLL)4, Enolase (Eno)-1, Forkhead box-p3-Exon2 (Foxp3-E2), miR-15a/16, and miR-4281. CNS0: special AT-rich sequence binding protein (Satb)1. CNS1: Smad2 and Smad3. CNS2: Foxp3, signal transducer and activator of transcription (STAT)5, runt-related transcription factor (Runx)1-core binding factor (CBF)-β, CREB, the methyl-CpG-binding domain (Mbd)2, the chromatin-remodeling complex tet methylcytosine dioxygenase (Tet)2, Eno-1, and Foxp3-E2. CNS3: Forkhead transcription factor of the O class (Foxo)1, Foxo3, and c-Rel. **(B)** Schematic representation of miRNAs modulating Foxp3 expression: miR-17, miR15a/16, miR-210, miR-24, and miR-31. Interferon regulatory factor (Irf)4.

lineage commitment (63–65). Demethylation of CpG motifs at the Foxp3 locus is correlated with stable Foxp3 expression in both human and mouse *ex vivo*-isolated Treg cells, while the same region is less demethylated in *in vitro*-induced Treg (iTreg) cells showing unstable Foxp3 expression (63, 66, 67). Moreover, it has been observed that IL-2-dependent stabilization of Foxp3 expression upon antigen stimulation significantly associates with demethylation of specific sequences at Foxp3 locus (68).

In the first stage of tTreg cell development, all double-positive thymocytes are highly methylated in the CNS2 region, and this epigenetic signature is also maintained in $CD4^+$ single-positive $Foxp3^-$ cells; a partial demethylation of CNS2 is observed in $CD4^+$ single-positive $Foxp3^+$ cells, and it becomes complete in mature Treg cells (69). Moreover, tTreg cells, which express Foxp3 notwithstanding CpG methylation, are not stable and lose their regulatory phenotype in the periphery (53). Toker and colleagues demonstrated that CNS2 demethylation, which begins in the early stage of tTreg cell development, occurs upon TCR stimulation and the thymic microenvironment is sufficient to enforce a regulatory identity (69). The methyl-CpG-binding domain (Mbd)2 protein binds to CNS2 and recruits both histone-modifying and chromatin-remodeling complexes, in particular tet methylcytosine dioxygenase (Tet)2, directly involved in CNS2 demethylation [**Figure 1**; (69–71)]. Both *in vitro* and *in vivo* murine Mbd2 deletion leads to a dramatic impairment of Treg cell suppressive function, due to a complete methylation of the CNS2 region (70). Nair and colleagues showed that in IL-$2^{-/-}$ early developing Treg cells, Tet2 downregulation is coupled with CNS2 region methylation. Culture of IL-2-deficient tTreg cells in the presence of recombinant (r)IL-2 and observation of rIL-2-dependent Tet2 expression strongly suggest a direct role of IL-2 in Tet2 maintenance (71). CNS3-deleted $CD4^+$ T cells are also unable to properly induce Foxp3, due to an impaired accumulation of mono-methylation of histone H3 at the Foxp3 promoter. Notably, Feng and colleagues observed that the impairment in Foxp3 induction is more evident in CNS3-deleted cells that received a weaker compared to cells that received a stronger TCR stimulation, thus indicating that increased TCR stimulation may partly compensate for the absence of CNS3 for the induction of Foxp3 expression (72). Recently, Kitagawa and colleagues have uncovered another regulatory CNS region, the CNS0, now considered a "super-enhancer" for Foxp3 induction in double positive thymocytes (73). CNS0 is bound by the special AT-rich sequence binding protein (Satb)1, a transcription factor that functions as a chromatin organizer, whose expression precedes Foxp3 protein appearance in Treg cell precursors, and whose deletion reduces Foxp3 expression and tTreg cell development [**Figure 1**; (73)]. Thus, Satb1 may be considered a "pioneer factor" during tTreg cell differentiation.

Modification of histones related to Foxp3 gene, such as histone H3 or H4 acetylation and mono-, di-, and tri-methylation of histone H3 at lysine (Lys) 4 (H3K4) or Lys 27 (H3K27), is also essential in Treg cell differentiation (74–76). Different families of enzymes catalyze these fundamental processes, which allow chromatin opening and transcriptional factor recruitment. In particular, the most important family of histone-modifying enzymes is composed of histone acetyltransferase (HAT), histone deacetylases (HDAC), histone methyltransferase (HMT), and histone demethylase (HDM) (**Figure 1**). These enzymes modify the N-terminal lysine or arginine residues: HAT and HDAC transfer or remove, respectively, acetyl groups to lysine residues; HMT and HDM transfer or remove one, two, or three methyl groups to/from lysine and arginine residues, respectively (77). *In vivo* HDAC3 deletion in mouse Treg cells causes lethal autoimmunity, due to an upregulation of several inflammatory-related genes, revealing HDAC3 role in promoting Treg cell development and functional activity (78). It has been reported that the methylation of H3K4 is catalyzed by a specific family of HMT, the mixed lineage leukemia (MLL) family (79). In particular, MLL4 binds to the Foxp3 promoter and 3′ untranslated region (UTR) and regulates epigenetic changes in H3K4, such as monomethylation of H3K4 (H3K4me1) (80, 81). Deletion of the MLL4-binding site by CRISPR-Cas9 technology in mice results in a decrease of Foxp3 induction in naïve $CD4^+$ cells during their development, with an increase of $CD4^+CD25^+Foxp3^-$ cells, demonstrating MLL4 requirement for the establishment of Foxp3 chromatin structure in Treg cell precursors (80).

The described finely tuned epigenetic regulation at Foxp3 locus (achieved by both DNA methylation and histone modifications) paves the way to a specific transcriptional program enforcing Foxp3 stable expression and the regulatory phenotype in Treg cells (56).

TRANSCRIPTIONAL REGULATION OF FOXP3

Several transcription factors bind either to the Foxp3 promoter or to the CNS regions to induce or maintain Foxp3 expression in tTreg cells [**Figure 1**; (56, 58)]. They are expressed early during Treg cell development upon TCR engagement and cytokine stimulation (i.e., IL-2, IL-15) and then bind specific DNA regions before Foxp3 protein expression (27, 36, 56). Forkhead transcription factor of the O class (Foxo)1 and Foxo3 proteins are two key regulatory determinants that induce Foxp3 expression by binding the promoter, CNS1, and CNS3 regions [**Figure 1**; (82–84)]. Foxo1 and Foxo3 function is tightly controlled through subcellular compartmentalization: conditions that promote Foxo nuclear localization are associated with Treg cell commitment, whereas after antigen or cytokine stimulation, these factors can be deactivated by phosphatidylinositol-3-kinase (PI3K)–Akt pathway phosphorylation that promotes their translocation from the nucleus into the cytoplasm, inhibiting the binding to Foxp3 regulatory regions (84–86). c-Rel, a member of the nuclear factor-κB (NF-κB) transcription factor family, is another important molecule, involved in Foxp3 control in tTreg cells; c-Rel deficient mice ($Rel^{-/-}$) show reduced levels of $Helios^+Foxp3^+$ Treg cells in the periphery, due to a defective thymic development, demonstrating that c-Rel is necessary for Foxp3 expression and tTreg cell generation (87). Mechanistically, c-Rel promotes Foxp3 expression through the formation of an enhanceosome, which encompasses the transcription factors c-Rel itself, p65, nuclear factor of activated T-cells (NFAT), Smad, and cAMP response

element binding protein (CREB) and induces the epigenetic changes at the Foxp3 locus, by recruiting the HAT p300 and CREB-binding protein (CBP) (88). c-Rel may also play a key role in pTreg cell generation through the binding of specific sites present in the CNS3 enhancer [**Figure 1**; (58, 89)]. Instead, Smad2 and Smad3 are essential for TGF-β-mediated induction of Foxp3 in pTreg cells through the binding to the intronic enhancer CNS1, important for Treg cell peripheral induction [**Figure 1**; (90)]. Recruitment of regulatory factors to Foxp3 locus also influences the expression of its splicing variants; in particular, it has been shown that the glycolytic enzyme enolase (Eno)-1 has an inhibitory effect on the transcription of the Foxp3 containing the exon 2 sequence (Foxp3-E2) through the binding to the promoter or to the CNS2 region of Foxp3 [**Figure 1**; (91)].

In tTreg cells, the complete demethylation of CpG islands is associated with the recruitment of several transcription factors, including signal transducer and activator of transcription (STAT)5, the runt-related transcription factor (Runx)1-core binding factor (CBF)-β, CREB, and Foxp3 itself [**Figure 1**; (92, 93)]. pTreg cells show a different signature compared to tTreg cells in terms of epigenetic modifications of Foxp3 and interaction with transcriptional factors that keep its expression stable (58, 66). Conflicting results are present in the literature concerning pTreg cell methylation pattern: some authors report demethylation of TSDR (63, 94); instead, others have shown its methylated status in pTreg cells (95).

In all, these findings concur to demonstrate that Treg cells express a unique epigenome and phenotype both in the thymus and in the periphery, finely regulated by specific enzymes and transcription factors.

MICRORNA-MEDIATED POST-TRANSCRIPTIONAL REGULATION OF FOXP3

MicroRNAs (miRNAs) are small (∼22 nucleotides in length) non-coding RNAs, which are part of the RNA interference silencing complex (RISC), and pair to complementary sequences usually present in the 3′ UTR of mRNAs, causing mRNA decay and block of protein translation, thus influencing physiological and pathological processes (96, 97). Mice in which miRNA maturation pathway is blocked suffer from a lymphoproliferative phenotype resembling the one observed in the absence of Foxp3 itself, indicating that Treg cell development necessitates the action of miRNAs (98–100). Of all miRNAs, deletion of miR-146a-5p results in a breakdown of immune tolerance and the development of a fatal spontaneous autoimmune disorder; highlighting it is the key positive regulator of Treg cell function (101, 102). On the other hand, Foxp3 positively regulates miR-155-5p and then the coordinated action of Foxp3 and miR-155-5p blocks key inducers of the effector lineage commitment, such as Satb1 and Zinc Finger E-Box Binding Homeobox (ZEB)2 (103–106). In other words, Foxp3 imposes a multi-layered suppression of specific genes in Treg cell by both direct binding to genetic regulatory elements and by induction of miRNAs that specifically target the 3′ UTR of the same genes. Several miRNAs have been hypothesized to directly target Foxp3 3′ UTR, thus decreasing its expression level and undermining Treg cell phenotype: it is the case of miR-31, miR-24, and miR-210 [**Figure 1**; (107, 108)]. In particular, modulation of miR-31 in Treg cells showed a significant regulation of Foxp3, and a luciferase reporter assay suggested that the miR-31 target sequence present in the 3′ UTR of Foxp3 may indeed make Foxp3 mRNA a direct target of miR-31 action [**Figure 1**; (107)]. Notably, in murine Treg cells, a Foxp3 ChIP assay reported significant recruitment of the transcriptional factor to the miR-31 promoter, that indeed contains a Foxp3 binding site, suggesting the existence of a tight regulatory loop between miR-31 and Foxp3 that may be crucial in regulating Treg cell homeostasis [**Figure 1**; (109)]. Other miRNAs can affect Treg cell physiology also by hampering the expression of proteins that co-operate with Foxp3. MiR-17, an individual mature miRNA of the miR-17-92 cluster, has the ability to directly target Eos, interferon regulatory factor (Irf)4, and Satb1, thus indirectly reducing Foxp3 transcriptional activity [**Figure 1**; (110)]. A very intriguing case is that of miR-15a and miR-16 (miR-15a/16). Their expression was found decreased in umbilical cord blood Treg cells and, while the overexpression of these miRNAs leads to a reversal of Treg suppressive activity, the knockdown induces regulatory functions. A luciferase assay suggests that miR-15a/16 expression may modulate Treg function through the specific molecular binding to Foxp3 mRNA (**Figure 1**). In addition, miR-15a/16 overexpression is also able to reverse the demethylation profile of the Foxp3 locus, known to discriminate Treg cells from activated Foxp3$^+$ Tconv cells (111), unveiling a novel mechanism by which miR-15a/16 regulate Foxp3 expression through the modulation of the DNA methylation/demethylation machinery [**Figure 1**; (111)].

Some miRNAs have the ability to associate with RNA polymerase II and TATA box-binding protein to bind the TATA box motifs of crucial genes, such as IL-2, insulin, and c-myc, and enhance their promoter activities and transcription initiation rate (112). MiR-4281, a miRNA specifically expressed in hominids, and the Foxp3 core promoter region share a remarkable complementary match, and the binding of the miRNA to the Foxp3 core promoter was computationally predicted and then experimentally validated: Treg cells induced in the presence of TGF-β, IL-2, and miR-4281 mimicking molecule were more stable and functional than those induced by TGF-β and IL-2 alone [**Figure 1**; (113)].

POST-TRANSLATIONAL MODIFICATION NETWORKS REGULATING FOXP3

Regulation of Foxp3 expression and function also acts at the protein level through covalent post-translational modifications, such as ubiquitination, acetylation, and phosphorylation of different amino acids. These processes influence Foxp3 subcellular localization, functional activity, and interaction with other proteins, mainly transcriptional activators or repressors. Protein ubiquitination is a process mediated by the concerted action of a large family of ligases (E1, E2, and E3) that catalyze

addition of ubiquitin peptides to lysine residues of a target protein regulating different processes, such as protein cellular trafficking or their degradation by the 26S proteasome [**Figure 2**; (114)]. High expression of deubiquitinase (DUB) ubiquitin-specific-processing protease (USP)7 is found in Treg cells and associates with the presence of Foxp3 in the nucleus. Treatment of cells with USP7 inhibitor results in reduced Foxp3 levels while ectopic expression of USP7 decreases Foxp3 ubiquitination and correlates with an increase of Foxp3 expression (114, 115). These findings suggest that Treg cell function can be regulated through a finely controlled mechanism consisting in the modulation of Foxp3 lysine residue ubiquitination. For example, during infection, a proper cellular response against foreign pathogens requires rapid downregulation of Treg cell number and function and the ubiquitination of Foxp3 is a signal for a rapid Treg cell switch-off (116). Moreover, in inflammatory conditions, the E3 ubiquitin ligase Stub1, expressed in response to danger signals, interacts with Foxp3 and, together with the chaperone Hsp70, catalyzes the K48-linked ubiquitination of Foxp3, leading to its downregulation [**Figure 2**; (116)]. The role of Stub1 in the regulation of Foxp3 level is reinforced by evidence that overexpression of Stub1 inhibits Treg cell suppressive activity and promotes a switch toward a T helper (Th)1-like phenotype, while, on the other hand, Stub1 knockdown is correlated with the inhibition of Foxp3 degradation (116).

A correlation between infection and increased ubiquitination of Foxp3 has also been reported during autoimmune diseases such as psoriasis (117). Defect in Treg cell function was already observed in psoriasis and several evidence suggested a strong correlation between infection and disease triggering (117), although a direct link had not yet been identified. Chen et al. reported that one of the links between infection and reduced Foxp3 stability in psoriasis is represented by the chemokine

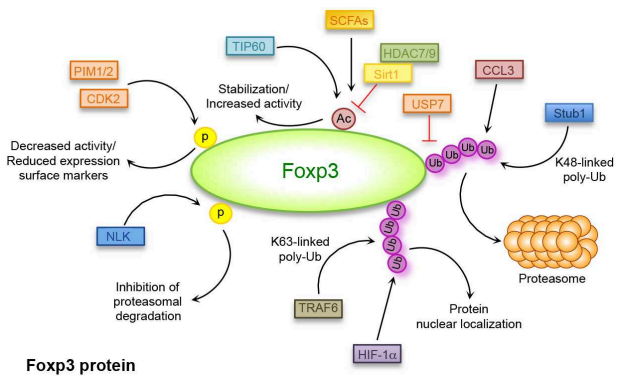

FIGURE 2 | Post-translational modifications regulating Foxp3 expression. Covalent post-translational modifications in Foxp3 amino acids: ubiquitination (Ub) mediated by deubiquitinase (DUB) ubiquitin-specific-processing protease (USP)7, STIP1 homology and U-Box containing protein (Stub)1, TNF receptor associated factor (TRAF)6, chemokine (C-C motif) ligand (CCL)3, and hypoxia-inducible factor (HIF)-1α; acetylation (Ac)/deacetylation mediated by Histone deacetylase (HDAC)7, HDAC9, tat-interactive protein 60 kDa (TIP60), sirtuin (Sirt)1, and short-chain fatty acids (SCFAs); phosphorylation (p) mediated by cyclin-dependent kinase (CDK)2, Nemo-like kinase (NLK), proto-oncogene serine/threonine-protein kinase (PIM)1, and PIM2.

(C-C motif) ligand (CCL)3, which is strongly induced during infections including the one from streptococcus (117). Patients suffering from psoriasis are characterized by high CCL3 serum concentration that strongly correlates with increased degradation of Foxp3 mediated by K48-linked polyubiquitination (**Figure 2** and **Table 1**). This phenomenon is mediated by the activation of the protein kinase B (PKB)α/Akt1 pathway (117), but the precise mechanism of how CCL3 and PKB induce polyubiquitination of Foxp3 in psoriasis is still under investigation.

Foxp3 polyubiquitination also plays a role in the regulation of Th17/Treg cell lineage fate. In hypoxic conditions, the hypoxia-inducible factor (HIF)-1, a metabolic sensor mediating the switch from oxidative metabolism to aerobic glycolysis (128), regulates the balance between Th17/Treg cells. HIF-1 promotes Th17 and inhibits Treg cell development through the binding to Foxp3 that induces its ubiquitination and subsequent degradation in the proteasome [**Figure 2**; (129)]. Addition of ubiquitin residues to a protein target is a signal not only associated to its degradation but to several biological processes (130). The K63-linked polyubiquitination, for example, is involved in the regulation of protein trafficking, signal transduction and protein–protein interactions (130, 131). Furthermore, Foxp3 nuclear localization, required for a proper Treg cell suppressive function, is supported by K63-linked polyubiquitination of Foxp3 mediated by the E3 ligase TNF receptor associated factor (TRAF)6 that interacts with Foxp3 catalyzing ubiquitination of the Lys 262 [**Figure 2**; (132)]. Also, it has been shown that in the absence of TRAF6, Treg cells display an altered suppressive function and the expression of a mutant form of Foxp3 resistant to K63 ubiquitination, unable to properly localize into the nucleus (132).

The activity of the Foxp3 transcription factor can also be regulated by other post-translational modifications, which include acetylation and deacetylation of specific lysine residues. This regulation occurs through the interaction of Foxp3 with lysine acetyl transferases (KATs also known as HATs) and lysine deacetylases (KDACs also known as HDACs) such as tat-interactive protein 60 kDa (TIP60), HDAC7, and HDAC9 [**Figure 2**; (133)]. Mass spectrometry analysis and structure-guided mutagenesis highlighted the presence of different acetylation sites in Foxp3 such as K31, K263, K268 (134), K250, and K252 (135). Acetylation of specific Foxp3 lysine residues augments Foxp3 stability and its ability to bind DNA (136) and activate specific effector functions [**Figure 2**; (134)]. This process competes with ubiquitination of the same sites and therefore increased acetylation inhibits the proteasomal degradation of Foxp3 reducing ubiquitination and *vice versa* (137).

It is well-known that sirtuin (Sirt)1-mediated deacetylation of Foxp3 associates with its reduced expression, as the result of an increased ubiquitination and subsequent degradation in the proteasome [**Figure 2** and **Table 1**; (138)]. Treatment of cells with nicotinamide, a Sirt inhibitor, results in reduced Foxp3 degradation together with increased Treg cell number and suppressive activity (138). The function of Sirt1 is regulated by the mammalian sterile 20-like kinase (Mst)1, which increases Foxp3 acetylation and promotes its activity both indirectly, by inhibiting the activity of Sirt1, and directly, by interacting with Foxp3 and preventing its binding to Sirt1 (139). As

TABLE 1 | Foxp3 post-translational modifications (PTM) and mutations accounting for the loss of Treg cell phenotype/function and associated diseases.

Alterations	Foxp3 PTM alterations/mutation domains	Effect	Treg cell-associated defect	Disease	References
Increased serum levels of CCL3	K48-linked polyubiquitination	Foxp3 degradation	Reduced Treg cell function	Psoriasis	(117)
Increased Sirt1 deacetylation	Reduced acetylation	Reduced Foxp3 expression	Impaired Treg cell number and suppressive function	Abdominal aortic aneurysm, Hashimoto's Thyroiditis, Grave's disease	(118–120)
Reduced TIP60 expression	Reduced acetylation	Reduced Foxp3 expression	Imbalanced Th17/Treg cell differentiation; impaired Treg cell proliferation	Rheumatoid arthritis	(121)
Germline mutations	FKH (A384T in Exon 11)	Altered interaction with TIP60	Impaired Treg cell suppressive function; impaired Foxp3 function; reduced Foxp3 stability	IPEX	(122–124)
Germline mutations	PRR (227delT, 303_304delTT in Exon 2)	Altered interaction with RORα and RORγt	Impaired Foxp3 function	IPEX	(125, 126)
Germline mutations	LZ (748_750delAAG, 750_752delGGA in Exon 7)	Impaired Foxp3 dimerization	Impaired Foxp3 function	IPEX	(127)

Chemokine (C-C motif) ligand (CCL)3, sirtuin (Sirt)1, tat-interactive protein 60 kDa (TIP60), forkhead domain (FKH), prolin-rich region (PRR), leucine zipper (LZ), RAR-related orphan receptor (ROR)α, immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX).

expected, Sirt1 deletion in Treg cells correlates with higher Foxp3 expression and Treg cell function and increases allograft tolerance (140). Foxp3 acetylation also regulates the balance between Th17 and Treg cell lineage differentiation (141). A key molecule involved in this process is the transcriptional coactivator with PDZ-binding motif (TAZ) that has a pivotal role in driving Th17 cell differentiation and inhibiting Treg cell development. Indeed, TAZ is a coactivator of the Th17-specific transcriptional factor RAR-related orphan receptor (ROR)γt and constrains Treg cell differentiation by decreasing Foxp3 acetylation (141). TAZ regulates Foxp3 acetylation by competing with it for the binding to TIP60, a HAT that mediates Foxp3 acetylation and inhibits its proteasomal degradation [**Figure 2**; (141)].

It is important to mention that gut immune homeostasis, influenced by the composition of the commensal microbial community, is maintained, at least in part, by mechanisms controlling Foxp3 acetylation [**Figure 2**; (142)]. It has been shown that microbial metabolites produced by commensal bacteria, such as short-chain fatty acids (SCFAs) butyrate and propionate, promote extrathymic Treg cell generation by increasing Foxp3 acetylation (142). Impaired acetylation of Foxp3 has been associated with the pathogenesis of several autoimmune diseases, such as rheumatoid arthritis (RA) and Hashimoto's Thyroiditis (HT) (**Table 1**). In RA, reduced expression of Foxp3 associates with imbalanced Th17/Treg differentiation and impaired Treg cell proliferation (121). The failure of Treg cell differentiation is secondary to reduced upregulation of TIP60 acetyltransferase, which determines lower level of Foxp3 acetylation during activation of T cells from RA-affected subjects [**Table 1**; (121)]. A study performed in HT subjects underlined low frequency of Foxp3$^+$ Treg cells, secondarily to reduced Foxp3 acetylation that leads to lower Foxp3 expression and impaired suppressive function [**Table 1**; (118)]. These alterations are paralleled by higher expression of the deacetylase Sirt1, suggesting a possible role of abnormal acetylation of Foxp3 in the pathogenesis of HT [**Table 1**; (118)]. A similar mechanism has also been described in subjects suffering from abdominal aortic aneurysm (AAA) where an autoimmune attack seems to have a pathogenic role (119). In these patients, a reduced function and percentage of Treg cells are associated with an increase of Sirt1 expression corresponding to decreased Foxp3 acetylation [**Table 1**; (119)]. Dysfunctions in Treg cell suppressive capacity associated with reduced acetylation of Foxp3 have also been identified in subjects with Graves' disease (GD). It has been shown that lower Foxp3 acetylation in GD is related to downregulation of miR-23a-3p that usually suppresses the expression of deacetylase Sirt1 with consequent alteration of Treg cell function [**Table 1**; (120)].

Foxp3 is also regulated through phosphorylation of specific sites and, depending on the phosphorylation site, it can be either activated or inhibited (143, 144). Foxp3 can be phosphorylated by the cyclin-dependent kinase (CDK)2 at four cyclin-dependent kinase (CDK) motifs (Ser/Thr-Pro) within the N-terminal repressor domain (**Figure 2**). These modifications are associated with reduced Treg cell suppressive ability, as CDK2-deficient Treg cells or mutations significantly increase Foxp3 and CD25 expression levels, together with a stronger Treg cell activity [**Figure 2**; (143)].

A novel TCR-mediated mechanism regulating Foxp3 phosphorylation involves the activation of the Nemo-like kinase (NLK), a serine/threonine protein kinase involved in regulation of cell proliferation and apoptosis (144). During T cell activation, TGF-β activates NLK that, in turn, binds and phosphorylates Foxp3 on multiple residues, inducing its deubiquitination and inhibiting the proteasomal degradation [**Figure 2**; (144)]. Also, under inflammatory conditions, Foxp3 is phosphorylated by the proto-oncogene serine/threonine-protein kinase (PIM)1 at the residue S422 in the C-terminal domain [**Figure 2**; (145)]. The pro-inflammatory cytokine IL-6 induces the expression of PIM1 that phosphorylates Foxp3, thus inhibiting Treg cell suppressive function and the expression of specific surface markers such as CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR) (145). Foxp3 phosphorylation is also mediated by the kinase PIM2 that physically interacts with Foxp3 and phosphorylates multiple sites in the N-terminal domain, inhibiting Treg cell stability and suppressive function [**Figure 2**; (146)].

These findings indicate that regulation of Foxp3 expression and the subsequent Treg cell function rely on several molecular processes; pharmacological manipulation of these pathways may open the way for novel immunological tools to control Treg cell function and immunological tolerance in immune-related disorders.

INDUCTION OF TREG CELL-ASSOCIATED TRANSCRIPTIONAL PROGRAMS

Foxp3 is the main defining factor of Treg cell lineage required for the induction of their functional and transcriptional program (147). However, gene-expression analysis of $Foxp3^+$ and $Foxp3^-$ T cells suggested that many Treg cell-specific genes are independent of Foxp3, thus changing the paradigm of Foxp3 as the only factor required for the establishment of Treg cell phenotype (11, 148). Treg cell lineage specification is indeed determined by the contribution of Foxp3-independent transcriptional programs that synergize with Foxp3 to ensure a Treg cell transcriptional signature (148). It has been observed that Treg cell fate determination is also influenced by the interplay of Foxp3 with elements related to TCR-mediated T cell activation, such as IL-2 and TGF-β signaling pathways (148, 149). Also, several Treg cell-specific molecules required for their suppressive activity—such as insulin-like 7, galectin-1, granzyme B, and Helios—are not under the transcriptional control of Foxp3 (10). In addition, Treg cell development, lineage stability, and suppressive function require CpG hypomethylation of specific sequences induced by TCR stimulation that could be fully achieved also without Foxp3 expression (53). However, although complete Treg cell differentiation requires additional transcriptional programs along with those induced by Foxp3, Foxp3 expression is crucial for survival of Treg cell precursor, Treg cell anergy, and lineage stability (28). In addition, Foxp3 induces and stabilizes the expression of genes encoding for Treg cell-specific surface markers such as CD73, CD39, TRAIL, and CTLA4 and acts as a repressor of effector cytokines induced by TCR activation such as IL-4, interferon (IFN)-γ, tumor-necrosis factor (TNF)-α, IL-17, and IL-21; this suggests that Foxp3 is necessary but not sufficient for the induction of Treg cell-specific signature (150).

The discovery of the key role of Foxp3 in the induction of Treg cell-specific transcriptional program gave rise to new questions about the mechanism of action of this transcription factor. Genome-wide analysis of Foxp3 target genes revealed that it acts both as a transcriptional activator and a repressor, through the binding to about 700 genes involved in TCR signaling pathway and maintenance of Treg cell functional programs (151, 152). Genes modulated by Foxp3 action are found both up- and downregulated in Treg cells in the thymus and in the periphery, thus changing the previous idea that Foxp3 mainly acts as a transcriptional repressor (151). However, only few Foxp3 target genes are characterized by a Foxp3 binding sequence, suggesting that the activation of Foxp3-induced transcriptional program is indirectly regulated through its cooperation with other cofactors (151). Biochemical and mass-spectrometric analyses revealed that Foxp3 might be associated with more than 360 proteins such as GATA3, NFAT, STAT3, Runx, and Foxp1, forming a large multiprotein complex of about 400–800 kDa (13). Some of these proteins are necessary for transcriptional regulation. Intriguingly, gene transcription of many Foxp3 cofactors is regulated by Foxp3 itself (13). Foxp3 is able to physically interact with sequence-specific transcription factors such as Runx1 and NFAT. The Runx1–Foxp3 complex suppresses IL-2 and IFN-γ production and induces the expression of Treg cell-specific markers such as CD25, CTLA4, and GITR (153). The key role of Foxp3 binding partners in sustaining Treg cell transcriptional program is supported by evidence showing that mutations of Foxp3 hampering its interaction with NFAT result in altered Foxp3 capacity to inhibit IL-2 secretion, upregulate CTLA4 and CD25 expression, and induce suppressor function of Treg cells (154). Moreover, the control of CTLA4 expression in Treg cells is mediated at least in part by the binding of Foxp3 to Foxp1. Indeed, a reduced binding of Foxp3 to the CTLA4 promoter region in the absence of Foxp1 has been reported (155). It has been suggested that Foxp3 interaction with NFAT prevents its binding with activator protein (AP)-1 fundamental for the induction of effector T cell responses (154). Another key partner of Foxp3 is Irf4, a transcription factor fundamental for Th2 cell differentiation. The interaction of Irf4 with Foxp3 promotes the expression of genes involved in the suppression of Th2 cell responses as suggested by the observation that Treg-specific ablation of Irf4 resulted in impaired capacity to inhibit the production of Th2 cytokines IL-4 and IL-5 (156). Foxp3 is also able to interact with GATA3, determining a reciprocal increase in their own expression; this interaction is involved in the control of Th2 responses by Treg cells (13). Foxp3 associates also with STAT3, and their interaction is lost in the presence of inhibitors of phosphorylation, suggesting that the binding is dependent on STAT3 phosphorylation state (157). The interaction between Foxp3 and STAT3 is involved in the control of Th17-mediated inflammation (13, 157). Moreover, Foxp3 is able to inhibit Th17 polarization also by

directly interacting, through its exon 2 region, with the master regulator of Th17 cell lineage RORγt. The binding of Foxp3 to RORγt prevents the activation of RORγt-mediated IL-17A transcription, thus inhibiting the polarization of cells toward a Th17 phenotype (125). Hench and co-workers identified another Foxp3 binding partner, Siva; the interaction between Foxp3 and Siva potentiates the repressive effect on NF-κB activity compared to that performed by Foxp3 alone (158). Foxp3-induced activation or repression of transcription of specific target genes is also maintained through the induction of epigenetic modifications thanks to the ability of Foxp3 to bind and recruit chromatin remodeling factors. Indeed, Foxp3 can associate with TIP60 and p300 and with HDAC7 that, by adding/removing acetyl groups to/from histones, modify chromatin accessibility to other transcription factors (133). Another factor involved in Foxp3-mediated epigenetic modification is Eos, a zinc-finger transcription factor of the Ikaros family with a key role in Foxp3-dependent gene silencing and induction of Treg cell suppressive function. Foxp3 interaction with Eos is essential for the repression of IL-2 promoter in Treg cells. It has been reported that Eos binds the carboxy-terminal binding protein (CTBP)1 that recruits factors involved in histone modifications and methylation of the IL-2 promoter (159). To repress the transcription of specific target genes upregulated upon T cell activation, Foxp3 also interacts and recruits the chromatin-modifying enzyme enhancer of zeste homolog (Ezh)2 to sustain the Treg-cell specific transcriptional program during inflammatory responses (160). Ezh2, the catalytic subunit of the polycomb repressive complex (PRC)2, is involved in chromatin condensation and gene transcription inactivation by promoting the tri-methylation of Lys 27 on the N-terminal tail of the histone H3 (H3K27me3) (161). The key role of Ezh2 in the induction of Treg cell stability and function is further confirmed by the observation that Ezh2 ablation in Treg cells results in the reduction of $Foxp3^+$ Treg cells in non-lymphoid tissues, inhibition of Treg cell capacity to control immune tolerance, and development of autoimmunity (162).

Although studies carried out in the last years have identified a plethora of Foxp3 cofactors, the precise mechanism by which Foxp3 regulates its target genes has not been fully understood. Kwon and co-workers proposed a model that hypothesizes the formation of two different multiprotein complexes in which Foxp3 can be alternatively integrated, located in different nuclear areas and with opposite transcriptional activity. More in detail, Foxp3 is in an active status, able to activate or repress the transcription of target genes, when part of a complex containing RelA-KAT5-IKZF2 is located at the center of the nucleus. On the contrary, when assembled in the complex containing Ezh2-IKZF3-YY1, Foxp3 is restrained at the nucleus periphery and has lower transcriptional function (163).

All these data suggest that the outcome of Treg cell transcriptional program is defined by an intricate balance between the formation of these two functional and non-functional multiprotein complexes. Moreover, Foxp3 controls the expression of several Treg cell specific genes, although several studies also highlighted the key role of some Foxp3-independent transcriptional programs in the induction of Treg cell signature.

FROM NAÏVE TO TERMINALLY EXHAUSTED TREG CELLS

It is well-known that the process of differentiation from naïve to memory cells is analogous in all T lymphocyte subsets, including Treg cells (3, 164, 165). Over the past years, the definition of naïve or memory T cell phenotype has been correlated with the expression of CD45RA or CD45RO in combination with other molecules such as CD62L, CD27, CD28, and C-C chemokine receptor 7 (CCR7) (166, 167). Accordingly, T cells may be separated in different groups: naïve T cells ($CD45RA^+CD62L^+CCR7^+CD27^+CD28^+$), central-memory T cells ($CD45RO^+CD62L^+CCR7^+CD27^+CD28^+$), effector-memory T cells ($CD45RO^+CCR7^-CD62L^-CD27^-CD28^-$), and terminally differentiated effector T cells ($CD45RA^+CCR7^-CD62L^-CD27^-CD28^-$) (167).

Several studies reported the highest frequency of naïve Treg cells in the cord blood, while they are about 6–10% of the total $CD4^+$ T cells in the peripheral blood of young adults and their frequency progressively declines with age (168–172). A study by Valmori et al. showed a subset of Treg cells that are $CD25^+CCR7^+CD62L^+CTLA4^+Foxp3^+$ and express the CD45RA molecule in human peripheral blood, named natural naïve Treg (NnTreg) cells. Analyses of telomere length and TCR excision circles in these cells revealed their early differentiation stage (170). In response to either TCR or autologous APC *in vitro* stimulation, NnTreg cells exhibit high proliferative capacity and partially downregulate CD45RA molecule while preserving the expression of the circulating marker CD62L. These findings suggest that NnTreg cells represent the precursors of the antigen-experienced Treg cell counterpart, which increases throughout life; moreover, the persistence of CD62L expression upon stimulation suggests that they are still able to migrate in the secondary lymphoid organs to accomplish their regulatory function (170). Accordingly, Booth et al. recently showed that memory Treg cell frequency in human subjects increases with age; however, a small fraction of naïve Treg cells expressing CD31, a marker identifying recent-migrating tTreg cells, is still observed in elderly people (>80 years) (173). These two subsets express different chemokine receptors to migrate into specific tissues. More in detail, human naïve Treg cells express high levels of the specific bone marrow homing CXC chemokine receptor type (CXCR)4, suggesting that this site represents a niche for naïve Treg cell maturation and proliferation, preceding their migration into target organs (173, 174).

Another classification of human Treg cell subpopulations has been proposed by Miyara and colleagues, on the basis of CD45RA and Foxp3 expression. More in detail, $CD45RA^+Foxp3^{low}$ subset identifies resting Treg (rTreg) and $CD45RA^-Foxp3^{high}$ activated Treg (aTreg) cells, while $CD45RA^-Foxp3^{low}$ represent non-Treg cells (3). In this study, the authors also revealed that rTreg and aTreg cells are both highly suppressive *in vitro*, compared to $CD45RA^-Foxp3^{low}$ non-Treg cells (3). Furthermore, *in vitro* and *in vivo* experiments showed that aTreg cells rise preferentially from TCR-stimulated rTreg cells, although a lower percentage may originate from $CD45RA^-Foxp3^{low}$ non-Treg cells (3, 175). Moreover, through a feedback mechanism, the expansion of

rTreg cells is under the control of aTreg cells, and this contributes to the maintenance of their balance (3). Of note, aTreg cells express high levels of CTLA4 and display the strongest suppressive capability with terminally differentiated Treg cell features (3).

Compelling evidence reports age-associated changes in number, phenotype, and function of Treg cells. Despite the thymic involution observed in elderly subjects, Treg cell frequency increases in blood overtime, suggesting a compensatory mechanism to balance the reduced thymic function (176–179). However, prolonged antigen stimulation of Treg cells throughout life may lead to exhaustion, a process characterized by loss of effector functions (180, 181).

Exhausted T cells are usually central or effector memory characterized by the expression of specific molecules, such as programmed cell death 1 (PD-1), lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin mucin 3 (TIM-3), and CTLA4. It has been described that human T cell exhaustion, resulting in loss of effector T cell function, is secondary to chronic antigenic stimulation, as those occurring during persistent infections or tumors (181). Furthermore, exhausted T cells are programmed to undergo apoptosis upon activation of the PD-1 pathway (182). Recent studies explored this end-differentiation process in depth also in Treg cells. In this context, Xiao and colleagues revealed a novel role for the molecule OX40 in the regulation of Treg cell homeostasis and function (183). OX40 is a secondary costimulatory molecule, a member of the TNFR superfamily, expressed on recently activated T cells. Several works reported that its expression and stimulation in Treg cells associate with altered suppressive ability (184–186). They showed that OX40 stimulation in Treg cells downregulates Foxp3 expression levels, leading to cell exhaustion, as confirmed by high PD-1 expression. This OX40-effect is controlled by IL-2, as exogenous addition of this cytokine could prevent Treg cell exhaustion (183). Moreover, Yang et al. suggested that liver kinase B1 (LKB1) protein, a bioenergetic sensor that controls cell metabolism and growth, is required to sustain the metabolic and immunological homeostasis of Treg cells necessary to prevent apoptosis and cell exhaustion. Indeed, loss of LKB1 in mature mice *Stk11*$^{fl/fl}$ Treg cells upregulates PD-1 and OX40 levels, suggesting a key role for LKB1 in the prevention of exhaustion (187).

Nowadays, the differentiation process of Treg cells is not completely explored and further studies are necessary to clarify in depth the molecular pathways involved in their differentiation status and how this could be influenced by Foxp3 expression.

FUNCTIONAL PLASTICITY AND REPROGRAMMING OF TREG CELLS

It is well-established that Treg cells maintain functional plasticity, modifying their transcriptional programs (34, 54). Several studies suggested that pro-inflammatory cytokines determine instability of Treg cell phenotype through the modulation of Foxp3 expression (188, 189) while others demonstrated that Foxp3 expression is particularly stable in Treg cells (94, 190). This debated issue was addressed by Hori and co-workers that proposed the "heterogeneity model" characterized by Treg cells having two different levels of commitment; on one side, there are Treg cells fully committed, resistant to conversion in other T cell subsets, while on the other, there are less committed Treg cells characterized by high degree of plasticity (191). This heterogeneity model has been demonstrated thanks to experiments showing that only a fraction of Treg cells adoptively transferred into lymphopenic mice loses Foxp3 expression and increases the production of effector cytokines becoming effector Th cells (192). Moreover, Miyara and colleagues showed that Foxp3$^+$ cells are composed of CD45RA$^-$Foxp3^{high} cells with suppressive capacity and non-suppressive CD45RA$^-$Foxp3^{low} cells, able to secrete pro-inflammatory cytokines (3). Interestingly, the suppressive capacity of CD45RA$^-$Foxp3^{high} cells correlated with the lower methylation status of the CNS2 region (13, 58, 193), important for the induction and stabilization of Foxp3 expression (58, 63, 67).

In the last few years, several reports questioned the concept that sustained expression of Foxp3 in Treg cells confers the stability of their suppressive function in different environmental conditions. Indeed, according to the transient flexibility model, Foxp3$^+$ Treg cells manifest a high degree of functional plasticity and, in response to various inflammatory stimuli, can be reprogrammed into effector-like T cells, with subsequent return to their specific phenotype upon resolution of inflammation (29, 194, 195). Treg cells can also be reprogrammed in Th17 cells *via* IL-6- and IL-1β-dependent signaling. This process is mediated by activation of STAT3, RORγt, and RORα that downregulate Foxp3 expression and promote Treg cells conversion into Th17 cells (126, 196). Tsuji and colleagues have also observed that Treg cells can differentiate into follicular Th (Tfh) cells in mouse Peyer's patches by losing Foxp3 expression. The selective differentiation of Treg cells into Tfh cells guarantees the interaction of Tfh with B cells. This phenomenon has been observed only in the gut and not in other lymphoid tissues, thus indicating a specific microenvironmental cue in Peyer's patches that promotes the differentiation of Treg into Tfh cells (197).

Metabolic signals could also control Foxp3 expression and Treg cell plasticity. In particular, HIF-1α is able to control the balance between Th17- and Treg-related programs reducing Treg cell development through the induction of Foxp3 degradation (129). It has also been reported that in response to the cytokine milieu, Treg cells undergo peripheral differentiation and specialization to support the specific effector function necessary to maintain immunological homeostasis. Indeed, in the presence of IFN-γ, Treg cells promote T-bet expression that in turn induces the upregulation of CXCR3 in a mouse model of inflammation. T-bet$^+$CXCR3$^+$Treg cells are able to migrate into inflamed tissues and inhibit Th1 responses. On the contrary, T-bet deletion in Treg cells determines uncontrolled Th1-mediated inflammation due to an impaired migration of Treg cells into the inflammatory site (198, 199).

The complex network that regulates Treg cell plasticity, including cooperative/counteractive transcription factors, external cues, and the stable transcription of Foxp3, represents

a great promise for future treatment of several immune-related diseases, including autoimmunity and cancer.

MODULATION OF FOXP3 DURING INFLAMMATION

A fine regulation of the intensity and duration of the immune response during inflammation is necessary to avoid tissue damage (200). Treg cells are crucial for maintaining immune homeostasis and prevent autoimmune diseases; on the other hand, their activity during an acute infection must be temporarily downregulated to allow adequate immune response against foreign pathogens. Moreover, increasing evidence suggests that, during inflammation, Treg cells may lose phenotypic stability and be converted into Teff cells with different phenotype (18). Several studies have been performed to understand whether inflammation drives this conversion affecting Foxp3 expression and stability. Inflammation induces post-translational modification of Foxp3, such as phosphorylation or its degradation through Stub1-mediated Foxp3 poly-ubiquitination [**Figure 3**; (116, 145)]. Furthermore, other mechanisms have been proposed through which inflammation regulates Foxp3 expression and stability. High amount of pro-inflammatory cytokines might favor the conversion and reprogramming of fully differentiated natural Treg (nTreg) and iTreg cells toward a Th17 phenotype during inflammation (196). In particular IL-6 has been involved in an active mechanism driving the reduction of Foxp3 expression [**Figure 3**; (201)]. The molecular events accounting for the conversion of Treg cells in "ex-Foxp3" Th17 cells by IL-6 involve the transcription regulator Id2, found upregulated in this cellular subset. Id2 overexpression during iTreg cell differentiation increases the expression of Th1- and Th17-related cytokines together with the reduction of Foxp3 mRNA and protein expression, suggesting a key role for this factor in the control of Treg cell stability (**Figure 3**). Upregulation of Id2 transcript and protein expression under inflammatory condition is promoted by the activation of STAT3, Irf4, and basic leucine zipper transcription factor, ATF-like (BATF), secondarily to the increased activity of the pro-inflammatory cytokines IL-1β and IL-6 (**Figure 3**). Id2 is able to inhibit the binding of the E-box binding transcription factor E2A to the Foxp3 promoter, resulting in a reduction of Foxp3 expression [**Figure 3**; (201)]. These findings unveil a key role for Id2 in the conversion of Treg in Th17 cells under inflammatory conditions.

Another mechanism leading to the reduction of Foxp3 expression during inflammation is mediated by the Deleted in breast cancer (DBC)1 factor, also known as p30 DBC or cell cycle and apoptosis regulator (CCAR)2 (202). Gao and co-workers recently reported that DBC1 is a Foxp3-interacting partner whose depletion in Treg cells results in the reduction of Foxp3 degradation and improvement of suppressive function in response to pro-inflammatory stimuli, such as IL-6 and TNF-α. The molecular mechanism underlying Foxp3 degradation during inflammation involves the activation of the caspase 8 degradation pathways in response to TNF-α stimulation. Indeed, treatment of cells with caspase 8 inhibitor during TNF-α treatment prevents Foxp3 degradation, thus suggesting a key role for caspase 8 in DBC1-mediated Foxp3 degradation [**Figure 3**; (202)].

As acute inflammation is accompanied by destruction of the surrounding host tissue, the highly tuned regulation of the duration and severity of the pro-inflammatory phase is necessary for organ/tissue regain of function (203). In this context, it has been recently shown that Treg cells can also activate specific compensatory mechanisms that augment their identity and function during inflammatory conditions (204). Indeed, the optimal expression and stability of Foxp3 require the complete demethylation of CNS2 sequence, also named TSDR,

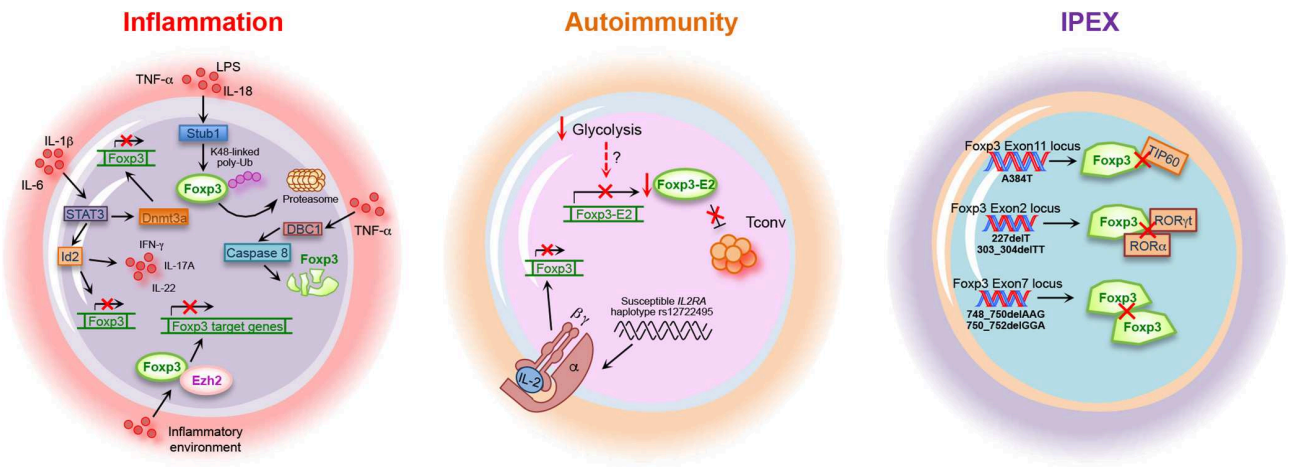

FIGURE 3 | Foxp3 alterations in inflammation, autoimmunity, and IPEX. Inflammation, autoimmune diseases and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome are associated with alteration of Foxp3 gene expression, stability, and function. Lipopolysaccharides (LPS), tumor necrosis factor (TNF)-α, interleukin (IL)-18, STIP1 homology and U-Box containing protein (Stub)1, Forkhead box-p3 (Foxp3), signal transducer activator of transcription (STAT)3, Id2, and interferon (IFN)-γ, Deleted in breast cancer 1 (DBC1) factor, enhancer of zeste (Ezh)2, DNA-methyltransferase 3a (Dnmt3a), Foxp3-Exon2 (Foxp3-E2), interleukin-2 receptor alpha chain (IL-2RA), tat-interactive protein 60 kDa (TIP60), and RAR-related orphan receptor (ROR)γt.

present in the first intron of the Foxp3 locus (66). Loss of Treg cell identity during inflammation has been associated with the increased expression of DNA-methyltransferase 3a (Dnmt3a)—secondarily to the activation of the IL-6-STAT3 pathway—able to methylate the CNS2, thus reducing Foxp3 expression stability [**Figure 3**; (65, 204)]. On the other side, prevention of Foxp3 downmodulation at the site of inflammation, such as during experimental autoimmune encephalitis (EAE), is mediated by the zinc finger protein Blimp1, which plays a key role in the control of Treg cell stability in inflamed non-lymphoid tissues by inhibiting the expression and function of Dnmt3a (204).

As previously described, the presence of pro-inflammatory cytokines is associated with downregulation of Foxp3 expression even in fully differentiated Treg cells. Moreover, it has been shown that several cytokines can also inhibit the peripheral differentiation of naïve $CD4^+$ cells into Treg cells in inflamed sites. Peripheral differentiation of Treg cells has a pivotal role in the control of immune homeostasis to prevent inflammation-induced tissue damage and takes place in particular in the GALT where it is promoted by GALT DCs *via* TGF-β and retinoic acid (205, 206). It has been reported that high levels of retinoic acid could inhibit the repression of Foxp3 expression in an inflammatory microenvironment overcoming the activity of the inhibitory cytokines IFN-γ and IL-4 (207). The molecular mechanism underlying the effect of retinoic acid in enhancing Treg cell generation involves the activation of CCAAT/enhancer-binding proteins (C/EBP)—in particular C/EBPβ–conferring resistance to the effect mediated by the inhibitory cytokines (208). C/EBPβ sustains Foxp3 expression in pTreg cells by binding to the methyl-CRE sequence in the Foxp3 TSDR. During the peripheral generation of Treg cells, the Foxp3 TSDR is gradually demethylated for the induction of the phenotype. The binding of C/EBP to the still methylated Foxp3 TSDR in the early stages of the induction process could represent a defense mechanism of Foxp3 against the action of the pro-inflammatory cytokines in a phase in which its expression is still unstable. C/EBPβ-transduced Treg cells express higher level of Foxp3, display an increased suppressive function with subsequent improvement of EAE and colitis when transferred in mouse models of diseases (208). All these data suggest the presence of a mechanism mediated by retinoic acid-dependent C/EBPβ activation, which promotes and supports the peripheral induction of Treg cells in an inflammatory environment and confirm the presence of mechanisms that activate and increase Treg cell suppressive activity after an inflammatory challenge.

It has also been observed that under inflammatory conditions and during Treg cell activation, Foxp3 induces changes in chromatin accessibility modifying gene expression of activated Treg cells in response to TCR signaling and inflammatory cues. Arvey et al. reported that, although after an inflammatory stimulus Foxp3 is able to bind the same DNA regulatory elements, it boosts its transcriptional repression thanks to the binding with the chromatin modifier Ezh2 [**Figure 3**; (160)]. In activated Treg cells, Foxp3 associates with Ezh2, favoring its deposition at Foxp3-bound loci; this leads to inflammatory-induced transcriptional repression of specific gene and induction of suppressive activity indispensable for Treg cells to inhibit inflammation (160).

Taken together, these data suggest that, although some inflammatory cytokines are involved in Foxp3 transcriptional repression, after an inflammatory challenge, signals mediated by TCR and cytokine receptors boost Treg cell suppressive activity to inhibit inflammation. The presence of a finely and timely regulated mechanism that controls Treg cell function in the course of an inflammatory response is also confirmed by a recent study showing that inflammation- and activation-induced changes in experienced Treg cells are gradually lost overtime to avoid global immunosuppression (209).

The full comprehension of inflammation-induced mechanisms controlling Foxp3 expression and Treg cell plasticity could be useful for the identification of new molecular targets to control autoimmunity or immunodeficiency.

FOXP3 STABILITY AND TREG CELL FUNCTION IN HUMAN AUTOIMMUNE DISEASES

Defects in Foxp3 expression, Treg cell development, and function lead to several human immune diseases (14). In particular, absence or lower expression of Foxp3 protein alters suppressive function, which is tightly regulated by Foxp3 itself and by its cooperation with several cofactors (13, 151, 210). Foxp3 is a crucial regulatory transcription factor, highly necessary to induce and stabilize the specific phenotype and functional characteristics of Treg cells (56, 211). Thus, Foxp3 mediates its function through the cooperation with several transcription factors (e.g., NFAT, Runx, GATA3, and STAT3), establishing a Treg cell-specific program that can either activate or repress defined target genes (13, 151, 154, 212). For this reason, impaired Foxp3 expression leads to *in vitro* and *in vivo* defective suppressive function, resulting in T-cell mediated autoimmunity (1, 210).

The main disorder associated to Treg cell loss of function is the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, characterized by germline mutations in the Foxp3 gene, both in non-coding and in coding sequences [**Table 1**; (2, 213)]. More than 60 Foxp3 point mutations have been identified so far, mainly situated in the regions encoding for the N-terminal proline-rich repressor (PRR) domain (e.g., E70H, T108M, and P187L), in the leucine zipper (LZ) domain (e.g., ΔE251), and in the DNA-binding, forkhead/winged helix (FKH) domain (e.g., R397W, I363V, and A384T) [**Table 1**; (122)]. All of them contribute to an impaired Treg cell development and suppressive function, leading to a systemic poly-autoimmune disease whose severity depends on the specific protein domain affected by the mutation (122). The most common IPEX mutation is the A384T, which selectively impairs Treg cell suppressive function [**Figure 3** and **Table 1**; (122)]. Indeed, Dhuban et al. recently revealed that the A384T mutation alters the protein domain required for the interaction between Foxp3 and TIP60, necessary for its functional activation and

stabilization [**Figure 3** and **Table 1**; (123, 124)]. They showed that restoration of the Foxp3–TIP60 interaction is able to re-establish the suppressive function of Treg cells and protect mice from the development of autoimmune disorders, such as colitis or arthritis (123). Recently, several mutations in exon 2 (e.g., 227delT; 303_304delTT) and in exon 7 of Foxp3 have also been reported (e.g., 748_750delAAG; 750_752delGGA) [**Figure 3** and **Table 1**; (127, 214–219)]. Foxp3 exon2 encodes the protein domain responsible for the binding to RORα and RORγt transcription factors (125, 126) while exon7 encodes for a sequence that is part of the LZ domain [**Figure 3** and **Table 1**; (127)]. Thus, IPEX subjects with mutations in these exons express a functionally defective Foxp3 protein, with a consequent impaired Treg cell suppressive activity.

However, besides these genetic alterations affecting Foxp3, loss of Treg cell function in autoimmune disorders is usually unrelated to Foxp3 mutations. This is the case in multiple sclerosis (MS) and type 1 diabetes (T1D) (220). Autoimmune pathologies are characterized by the selective destruction of *self*-tissues by autoreactive T lymphocytes, due to the lack of peripheral tolerance secondary to impaired Treg cell homeostasis and/or function (14, 221, 222). Several groups showed a defective number of $CD4^+CD25^+Foxp3^+$ Treg cells in the peripheral blood of relapsing–remitting (RR)-MS subjects (223–225). Moreover, Treg cell number inversely correlates with the expanded disability status scale (EDSS), which reflects the disability grade, thus demonstrating a strong association between Treg cell frequency and disease severity in RR-MS (127). In addition, Fletcher and colleagues found that a specific subset of Treg lymphocytes, $CD39^+Foxp3^+$, is reduced and functionally impaired in MS subjects. More in detail, they showed in healthy subjects that the $Foxp3^+$ Treg cells that also express the ectonucleotidase CD39 are able to suppress IL-17 production by $CD4^+$ T cells, probably removing extracellular ATP, which is necessary to promote differentiation and function of Th17 cells (226, 227). Thus, impaired expression of Foxp3 and CD39 proteins and subsequent reduced frequency and regulatory ability of this Treg cell subset could promote MS through an increase of pathogenic Th17 cell frequency (226).

Treg cell frequency has also been analyzed in brain tissue and cerebrospinal fluid (CSF) of MS subjects. Several studies showed that the absolute number of $Foxp3^+$ cells is rather low or undetectable in MS brain lesions despite Treg cells being present in the CSF of MS subjects at higher frequency than in peripheral blood (228–230). However, the well-described functional impairment of peripheral Treg cells in MS subjects makes their higher frequency in CSF useless. The reduced frequency and suppressive ability described in Treg cells from RR-MS subjects are most likely due to lower Foxp3 expression, both as mRNA and protein (223, 224, 231). It is well-known that several isoforms of Foxp3 exist in human Treg cells, due to alternative splicing (127). In this context, Foxp3 splicing variants containing the sequence corresponding to the exon 2, Foxp3-E2, are considered necessary for a proper Treg cell function (127, 232, 233). Work by our group and others revealed the importance of the Foxp3-E2 in Treg cell suppressive function (91, 125, 234–236). Indeed, in healthy individuals, this subset expresses higher level of Treg cell-associated markers (e.g., CTLA4, PD-1, Ki67, and GITR) compared to the counterpart expressing the other splicing variants, confirming a major regulatory role (91, 236). We also showed that naïve-to-treatment RR-MS subjects have a reduced frequency of Foxp3-$E2^+$ Treg cells (**Figure 3**). Moreover, the induction of Foxp3-$E2^+$ iTreg from Tconv cells is also impaired due to a deranged glycolytic engagement affecting the transcriptional regulation of Foxp3-E2 (91). This defect in iTreg cell frequency and function has also been confirmed in recent-at-onset T1D subjects, demonstrating that impaired induction of Foxp3-E2 could be a common phenomenon in autoimmunity (**Figure 3**) (91). Accordingly, reduced function of Treg cells has also been shown in T1D individuals (237, 238). A recent study revealed that Treg cells from T1D subjects have an impaired activation, as demonstrated by the reduced frequency of the $CD4^+CD25^+Foxp3^+CD45RO^+$ memory Treg cell compartment (239). This impaired memory Treg cell frequency associated with reduced residual pancreatic β-cell function, evaluated as C-peptide secretion (239). Moreover, Ferraro et al. demonstrated that, although Treg cell frequency is similar to healthy controls, the expression of Foxp3 is reduced in Treg cells infiltrating the pancreatic lymph nodes (PLNs) of diabetic patients, with an increase of the Th17 counterpart. As demonstrated by the analysis of the TSDR performed in $CD4^+$ T cells, the generation of "ex-Treg cells" in the PLNs of T1D patients is the result of Foxp3 protein instability (238). In all, these findings suggest impaired Treg cell activation and deranged suppressive function in T1D, as observed in MS.

Although the failure in central and peripheral tolerance secondary to reduced Treg cell frequency or function is the most common hypothesis for the development of autoimmunity, the origin of the loss of *self*-tolerance is still under debate (14, 240). Several evidence suggest that the IL-2/IL-2R pathway is necessary for Treg cell survival and functional activity, by promoting the expression of Foxp3 and other regulatory markers, such as CTLA4 (40, 41, 241–243). In this context, it has been shown that defects in IL-2/IL-2R signaling in Treg cells profoundly contribute to the development of autoimmunity since IL-2 has a key role in the control of Foxp3 expression in $CD4^+CD25^+$ cells, through the phosphorylation of STAT3 and STAT5 proteins (244–246). Moreover, deregulation of IL-2 pathway leads to altered proliferation and reduced Foxp3 expression in Treg cells from RR-MS subjects, and these parameters inversely correlate with disease clinical score (EDSS) (247). Aberrant IL-2 receptor (IL-2R) signaling has also been demonstrated in Treg cells from T1D subjects, and the T1D association in the gene region encompassing IL-2RA was firstly discovered by Vella et al. [**Figure 3**; (248, 249)]. More in detail, Garg and colleagues revealed that the presence of an autoimmune disease-associated IL-2RA haplotype in T1D subjects associates with reduced IL-2 responsiveness in Treg cells, which correlates with a lower Foxp3 expression and impaired suppression of $CD4^+CD25^-$ T cells [**Figure 3**; (250)]. In particular, it has been shown that nTreg cells isolated from T1D subjects and cultured with IL-2 show reduced Foxp3 protein stability compared to those isolated from healthy controls. This is due to a reduced IL-2

responsiveness, consequent to impaired activation of the IL-2R/STAT5 pathway (251).

Taken together, these findings underline the key relevance of Foxp3 in the control of Treg cell function and how its related defects concur to autoimmune disease pathogenesis and progression.

CONCLUDING REMARKS

Treg cells represent a subset with a TCR bias toward *self*-epitope MHC recognition; thus, its plasticity and reprogramming into Teff cells has the potential to unleash autoimmunity. The inflammatory environment influences Foxp3 stability, in turn affecting Treg cell identity and redirecting differentiation into Teff cells. Since Treg cells acquire effector functions through the modulation of transcriptional networks controlling Foxp3 expression, its direct regulation together with the control of its cofactors represent a key immunological strategy for the treatment of autoimmune diseases. In this context, several HDAC or DNMT inhibitors (63, 252) and specific immune modulators able to neutralize proinflammatory cytokines [e.g., anti-IL2, anti-TNF-α (253, 254)] have been used to restore Foxp3 expression and Treg cell suppressive function *in vitro*, representing promising tools for future clinical trials in human autoimmune disorders. Understanding the molecular underpinnings of Foxp3$^+$ Treg cell stability and the dynamics of physiological Treg cell function will shed light into their pathological dysregulation and delineate novel therapeutic strategies to halt autoimmunity.

AUTHOR CONTRIBUTIONS

VD conceived the work. AC, FC, SB, PC, and VD wrote the manuscript. AC, FC, SB, CF, GM, FD, and PC conceived the artwork and performed bibliographical research. MG, PC, and VD supervised the writing.

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The Impact of Dietary Components on Regulatory T Cells and Disease

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The rise in the prevalence of autoimmune diseases in developed societies has been associated with a change in lifestyle patterns. Among other factors, increased consumption of certain dietary components, such as table salt and fatty acids and excessive caloric intake has been associated with defective immunological tolerance. Dietary nutrients have shown to modulate the immune response by a direct effect on the function of immune cells or, indirectly, by acting on the microbiome of the gastrointestinal tract. FOXP3$^+$ regulatory T cells (Tregs) suppress immune responses and are critical for maintaining peripheral tolerance and immune homeostasis, modulating chronic tissue inflammation and autoimmune disease. It is now well-recognized that Tregs show certain degree of plasticity and can gain effector functions to adapt their regulatory function to different physiological situations during an immune response. However, plasticity of Tregs might also result in conversion into effector T cells that may contribute to autoimmune pathogenesis. Yet, which environmental cues regulate Treg plasticity and function is currently poorly understood, but it is of significant importance for therapeutic purposes. Here we review the current understanding on the effect of certain dietary nutrients that characterize Western diets in Treg metabolism, stability, and function. Moreover, we will discuss the role of Tregs linking diet and autoimmunity and the potential of dietary-based interventions to modulate Treg function in disease.

Keywords: diet, microbiome, Treg—regulatory T cell, autoimmunity, environmental factors

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INTRODUCTION

An appropriate balance between pro- and anti-inflammatory immune responses is required to protect organisms from invading pathogens and tumor development without incurring in autoimmune and allergic diseases. While different cell populations with anti-inflammatory activity have been identified, CD4$^+$FOXP3$^+$ regulatory T cells (Tregs) are the most well-defined. FOXP3 transcription factor determines Treg cell lineage and is essential for appropriate immune homeostasis. Loss-of-function mutations in *foxp3* lead to fatal immune disorders in humans (IPEX) (1, 2) and mice (Scurfy phenotype) (3).

Tregs suppress innate and adaptive immune responses using a broad array of molecular mechanisms which e.g., involve cell-contact dependent mechanisms (4), the release of soluble factors (5, 6), deprivation of growth factors (7), induction of apoptosis of target cells (8), and ATP hydrolysis and adenosine production (9, 10). Although there is versatility in the Treg response that allows for a specialized response according to the environment, the anatomical location, and the type of the cell to suppress (11, 12), increasing evidence suggests lack of Treg stability as a culprit of autoimmunity (13). Tregs isolated for instance from T1D (14, 15), MS (16–19) and SLE

(20) patients showed acquisition of pro-inflammatory functions and reduced suppressive potency *in vitro*.

Whereas, genetic factors clearly predispose to autoimmune development, the dramatic increase in the incidence of autoimmune diseases in Western countries suggests Western lifestyle patterns as important triggers of disease [reviewed in (21, 22)]. A variety of factors have been proposed to favor autoimmune development such as decrease pathogen exposure, smoke, hormones, stress, pollutants, dietary components and obesity (23–27). Moreover, increasing data highlight the complex interplay between nutrition, metabolic state and the immune response. Caloric restriction ameliorates disease severity and increases the lifespan in experimental animal models of inflammation and autoimmunity (28–30). By contrast, obesity is one of the most consisting factors that predispose for autoimmunity, having being linked with MS (31), T1D (32), psoriasis (33), and Chron's disease (34) (**Figure 1**). In addition, diet alters the gut microbial composition. Gut bacteria and their metabolites regulate pro-inflammatory and regulatory T cell responses in the gut, which could exert systemic effects in the individual (35–37).

Although there are many other cell types and environmental factors involved in triggering autoimmunity, given their crucial role in disease regulation, we will summarize the evidence provided by experimental and epidemiological studies associating nutrition, regulatory T cell function and autoimmunity.

Treg REGULATION AND HETEROGENEITY

We and others have shown before that different cell subsets can be distinguished within the pool of Tregs (9, 38). Recent immune phenotyping by mass cytometry and single cell transcriptomic analysis have further demonstrated the heterogeneity of the FOXP3$^+$ Treg population (39, 40). Therefore, these technologies could potentially aid in the identification of novel markers involved in Treg function, stability and migration and in gaining a better understanding of Treg biology. Tregs are typically categorized according to their origin into two subsets; those that develop in the thymus (tTregs) as a distinct cell lineage, and those induced from CD4$^+$CD25$^-$FOXP3$^-$ naive T cells in peripheral tissues (pTregs). *In vitro*, FOXP3$^+$ Tregs can also be generated from CD4$^+$FOXP3$^-$ T cells by e.g., culturing them in the presence of TGF-β, IL-2, and anti-CD3 stimulation (41, 42) being generally named as iTregs, although their functional activity is not well-defined in humans.

FOXP3 is regulated at transcriptional and post-transcriptional level in response to environmental cues [reviewed in (43)]. Demethylation at specific regions of the *foxp3* locus is pivotal for regulating FOXP3 expression in different Treg subsets (44). Moreover, distinct FOXP3 splicing variants have been described in humans (45–49) and variations in their relative expression are present in autoimmune disease patients (50–54), suggesting a link between FOXP3 post-transcriptional regulation and autoimmune pathogenicity.

Signals driven by the cytokine milieu (55–59), co-stimulatory molecules (60–62) and the strength of the TCR signaling (63–65) allow Tregs to adapt to the immune environment through e.g., changes in FOXP3 expression. Several studies have shown that, under certain inflammatory conditions, some Tregs secrete pro-inflammatory cytokines and lose their suppressive function (13, 66–72). Interestingly, phenotypically distinct Treg subsets in humans and mice have been described that mirror CD4$^+$ Th cell populations by specific co-expression of chemokine receptors, cytokines, and lineage specifying-transcription factors classically associated with Th cells (18, 73–76). The acquisition of Th-specific markers may allow Tregs to co-localize and regulate particular Th cell subsets *in vivo* (76). However, it might also be an indication of loss of function. Indeed, an increase in IFN-γ-producing Tregs has been associated with e.g., T1D, MS and autoimmune hepatitis (15, 18, 77). Also, the frequency of Tregs expressing IL-17 is increased in e.g., human patients with psoriasis, IBD and RA (62, 78–83). These data suggest that some Th-like Tregs may lose their ability to suppress immune responses and, instead, may contribute to autoimmunity.

Additionally, Tregs show phenotypical differences depending on which tissue they reside in, with the best non-lymphoid-tissue Treg populations described being those residing in visceral adipose tissue (VAT), skeletal muscle, colonic lamina propria and skin [reviewed in (84–86)]. In general, tissue-resident Tregs are characterized by higher frequency, self-antigen TCR repertoire with clonal micro-expansion, and a specific transcriptional profile different from Tregs in lymphoid organs (87–90). Moreover, by the use of single cell transcriptomics it was revealed that Tregs are highly homogenous within each tissue (40). These distinct phenotypes allow for cell accumulation in specific tissues and dedicated function within the microenvironment [reviewed in (84)].

The existence of multiple Treg subsets with specialized function dependent on environmental signals shows the complexity of Treg biology, but it also makes Treg plasticity and function susceptible to pharmaceutical intervention. Whether changes in diet or microbial composition associated with a Western lifestyle can control Treg function is being actively studied.

OBESITY AND VAT Tregs

Excessive calories are stored as fat in adipose tissue, which also acts as an "endocrine organ" releasing pro-inflammatory adipokines and cytokines such as TNF-α, IL-6, IL-1β, and leptin, resulting in systemic low-degree chronic inflammation (91, 92) (**Figure 1**). Multiple immune cell types reside in the adipose tissue and play a role in inflammation and metabolic dysregulation (87, 93–99). In particular, obese mice display a dramatic reduction in Treg numbers specifically in adipose tissue, but not in other fat depots, nor in other non-lymphoid tissues and spleen or lymph nodes (87, 100). Cytokines and adipokines may be involved in controlling Treg fluctuations in obese VAT. Obesity positively correlates with IL-6 and IL-17 expression in mice and humans (101, 102). IL-6 promotes

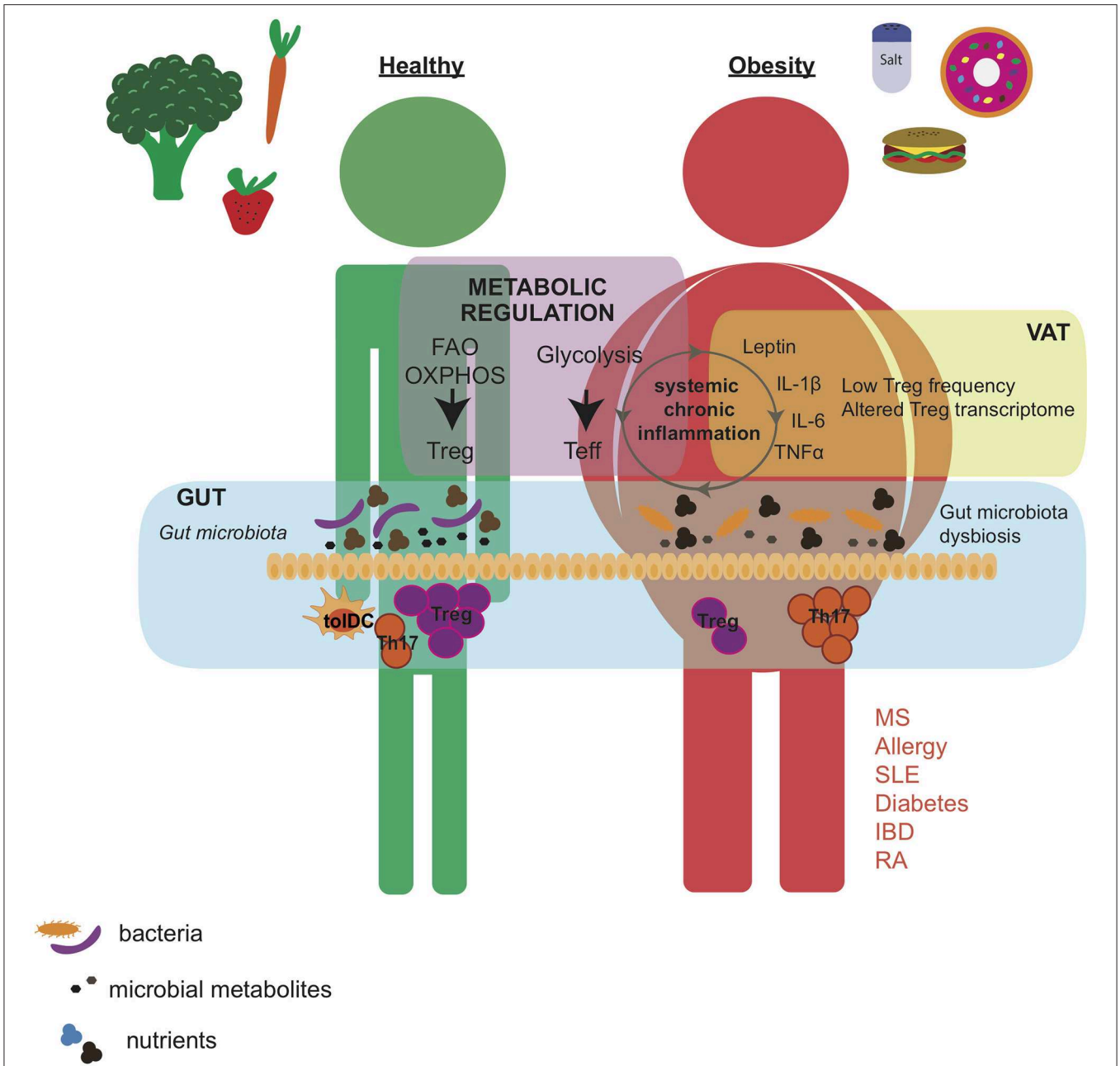

FIGURE 1 | Western diet affects gut microbial composition and induces low-degree chronic inflammation that alters the metabolic status of the individual. Healthy diet supports the growth of bacterial species that, by the production of immunomodulatory metabolites, promotes Treg induction over Th17 cell development in the intestine. By contrast, western diet, characterized by high caloric intake, and high levels of salt and cholesterol, leads to obesity, increased secretion of pro-inflammatory adipokines and cytokines, and altered gut microbial composition. These changes are associated with an altered Treg phenotype and higher Th17 cell differentiation in the VAT and intestine. Importantly, Treg and Teff cells developed in the gut and VAT have a systemic effect and may contribute to exacerbation of autoimmune pathogenicity. Moreover, the frequency and function of Tregs may also be regulated by metabolic pathways such as FAO, OXPHOS, and glycolysis that depend on the nutritional state of the individual. VAT, visceral adipose tissue; Teff, effector T cell; FAO, fatty acid oxidation; OXPHOS, oxidative phosphorylation; MS, multiple sclerosis; SLE, systemic lupus erythematosus; IBD, inflammatory bowel disease; RA, rheumatoid arthritis.

Th17 over Treg development (103) and obese-induced Th17 cell expansion was correlated with exacerbated disease symptoms in autoimmune disease models of experimental autoimmune encephalomyelitis (EAE) and colitis (102, 104). Leptin favors Th1 responses (105–109) and Th17 differentiation (110), but inhibits Treg proliferation (111). Moreover, leptin deficient mice showed a decrease in pathogenic inflammation in most of experimental models of IBD (112), RA (113, 114) and MS (108,

109). Similarly, relapsing-remitting MS patients also displayed an inverse correlation between frequency of Tregs and serum leptin levels (115), indicating that leptin may act as a link between obesity, Treg numbers and immunological tolerance.

In addition to differences in frequency, it was demonstrated that VAT-Tregs isolated from genetically promoted (leptin-deficient) or diet-induced (high fat chow) insulin-resistant mouse models of obesity have an altered transcriptional signature compared to lean mice (116). Phenotypical changes driven by obesity highlight the adaptability of VAT-Tregs to metabolic perturbations and suggest that obesity might alter Treg plasticity. Although most of the molecular mechanisms still need to be elucidated, it has been shown that excessive caloric intake leads to a dysregulation of intracellular nutrient-energy-sensing pathways and metabolic overload in immune cells (117, 118).

METABOLIC REGULATION OF Tregs

Cellular metabolism regulates cell development, proliferation and function and is controlled by environmental cues and nutrient availability (119, 120). Tregs have a specific metabolic profile, which is mainly dependent on mitochondrial metabolism through fatty acid oxidation (FAO) or pyruvate dependent oxidative phosphorylation (OXPHOS) (121–125). mTOR, one of the main pathways linking nutritional availability with cellular activity, promotes glycolysis (126, 127) and regulates differentiation of Th1, Th17, and Tregs (128–130). Studies showed that *in vitro* over-activation of mTOR, by culturing in media containing high concentration of nutrients or leptin, impaired Treg proliferation and the induction of FOXP3 expression (131). Treatment with rapamycin or neutralizing anti-leptin mAb reversed this effect and resulted in increased Treg frequencies and lessened EAE severity. However, continuous treatment with rapamycin or genetic mTOR silencing impeded Treg proliferation in the long term *in vivo* (131). Hence, periods of high and low nutrient levels, required for oscillatory changes in mTOR activity, may be necessary for Treg homeostasis and immunotolerance [reviewed in (132)].

Deletion of PTEN, a negative regulator of PI3K, also contributes to Treg regulation by enhancing glycolysis, decreasing FOXP3 expression and inducing the generation of effector T cells (133, 134). Additionally, the metabolic sensor LKB1 acts through AMPK promoting OXPHOS over glycolysis, and its deletion on Tregs led to alterations in cellular metabolism and the development of autoimmune diseases associated with dampened FOXP3 expression (124, 135). On the other hand, AMPK is considered an antagonist of mTOR activity with the ability to promote FAO (136, 137). Berod et al. showed that deletion of ACC1, a key enzyme in fatty acid synthesis, promoted AMPK activity in $CD4^+$ T cells leading to increases in FAO and Treg development, and ACC1 inhibition under EAE conditions improved disease severity by increasing Treg/Th17 ratio (138).

Vitamins and indoles also modulate Treg function (59). For example, retinoic acid (vitamin A metabolite) acts in conjunction with TGF-β promoting the induction of Tregs from naive T cells and stabilizing FOXP3 expression, which prevented their conversion into Th1/Th17 cells in the presence of IL-1β/IL-6 (139, 140). Calcitriol, a vitamin D metabolite, enhanced the growth of Tregs (141, 142). Vitamin C has been found to increase the generation of $FOXP3^+$ iTregs on alloantigen-specific Treg induction cultures and to cause a pronounced TSDR demethylation, resulting in an elevated FOXP3 stability (143). Interestingly, vitamin C treatment may act in a distinct manner on tTreg and iTreg function. A recent study by Oyarce et al. showed that tTreg cells pretreated with vitamin C before coculturing with effector $CD4^+$ T cells did not enhanced Treg ability to suppress T cell proliferation regardless of their increased FOXP3 expression. By contrast, *in vitro*-induced iTregs generated in presence of vitamin C showed improved suppressive capacities (144).

Metabolites associated with aryl hydrogen receptor (AHR) also control Treg function. Kynurenine is important for the generation, expansion and function of Tregs (145–147) and indole-3-carbanole (I3C) and 3,3′-diindolylmethane (DIM) promoted Treg infiltration to the CNS under EAE conditions, improving disease severity, and progression (147).

These data highlight the potential of targeting Treg metabolism to ameliorate autoimmune disease progression. However, more research regarding the therapeutic level of such modulation still has to be conducted. Besides, the limitations associated with studying Treg metabolism, due to their plasticity, culturing method, and biological source prompt a big challenge in the field.

THE EFFECT OF SALT IN Treg PLASTICITY

Increased intake of salt that is also common in Western diets has been linked with cardiovascular disease (148, 149) and autoimmunity (22, 150–154). Although the specific mechanisms are still being revealed, several studies in murine EAE and colitis models have demonstrated that elevated NaCl intake could exacerbate disease by promoting the induction of pathogenic Th17 cells via the SGK1-FOXO1 pathway (35, 36, 155). Besides, Wu et al. have recently described a direct effect of SGK1-FOXO1 in controlling Treg function, such as SGK1 deficiency in Tregs protected mice from the development of autoimmunity (156).

High-salt also induced secretion of IFN-ɤ and repressed IL-10 expression in Tregs, which resulted in impaired suppressive function *in vitro* and *in vivo* (155, 157, 158). In mice fed with high-salt diet, Tregs secreted more IFN-γ and failed to control colitis and xenogeneic GvHD, which was dependent on SGK1 signaling (157). IFN-γ-secreting $FOXP3^+$ Tregs with reduced IL-10 expression have been found in MS and T1D patients and are thought to contribute to disease (15, 18, 158). Interestingly, the imbalance between IFN-γ- and IL-10-expressing-Tregs in MS patients was also observed when Tregs from healthy donors were exposed to high-salt *in vitro* (158), suggesting that a high-salt environment could skew Tregs toward a dysfunctional state. Moreover, PTGER2 and β-catenin appeared as upstream regulators of the SGK1-FOXO1 axis in response to high-salt concentration, and constitutive expression of active β-catenin in Tregs caused the development of Scurfy-like autoimmunity

(158). Interestingly, stabilized active β-catenin has been also found in MS patients (158), suggesting that similar regulation may exist in humans. Recently, Luo et al. have proposed that, while high-salt alters the function of tTregs, it has no effect on pTregs or iTreg, which maintained unaltered transcriptional signature and stable FOXP3 expression, cytokine profile and suppressive function under high salt conditions (159). However, Wu et al. reported higher FOXP3 expression and regulatory function in iTregs and pTregs that lack SGK1 function (156), indicating that iTregs and pTregs could also be affected by high-salt via SGK1-FOXO1 axis. More studies are therefore required to clarify the role of high salt in Treg subpopulations.

Based on these findings, multiple studies have attempted to study the relation between salt intake and autoimmune disease in humans. In MS patients, Farez et al. have reported a positive correlation between disease activity and increased dietary sodium intake (150). Moreover, higher sodium concentration was observed in acute MS lesions than in chronic lesions (160). However, studies in larger cohorts have shown no significant correlation between salt consumption and the risk of MS development (161) or disease severity (162). Limitations in accurate measurement of NaCl levels and in the identification of the specific effect of salt independently of other dietary components may account for these controversial results [as discussed in (163)]. Since moderate increases in salt intake has proven to affect human immune cells, including T cells *in vivo* (26, 37), more specific analysis are needed to establish the role of NaCl in human autoimmune disease.

THE GUT MICROBIOTA AFFECTS DISEASE-CONTROLLING Tregs

The human gastrointestinal tract is the major reservoir of microorganisms including bacteria, microeukaryotes, archaea, and viruses, all of which collectively constitute the commensal microbiota (164). Extensive research has demonstrated the intimate crosstalk between commensal microbiota and immune balance. Tregs residing in the intestine are critical for maintaining intestinal immune homeostasis (165–167). Increasing evidence shows that Tregs in the colonic lamina propria are mostly peripherally-induced and depend on microbiota-derived signals for proper development and function (90, 168, 169). In fact, germ-free mice or antibiotic-treated mice show a substantial reduction in colonic Treg frequency (168, 170, 171). Interestingly, induction of pTregs in the small intestine appears more dependent on dietary antigens than on microbial signals (172). By investigating germ-free mice fed on an antigen-free diet, Kim et al. elegantly showed reduction in pTreg numbers in small intestinal lamina propria compared to germ-free mice fed on conventional diet. Importantly, pTregs residing in small intestinal lamina propria suppressed immune responses against dietary antigens (172).

Haghikia et al. elegantly demonstrated that dietary fatty acids profoundly impact T cell subset differentiation in the gut, which had a subsequent impact on central nervous system autoimmunity. The authors showed that SCFAs increased Treg proliferation while long-chain fatty acids (LCFAs) supported Th1 and Th17 differentiation in the gut, which had a significant effect in EAE severity (173). The short-chain fatty acid (SCFA) butyrate, derived from fermentation of dietary carbohydrates by gut microbiota, is an important promotor of colonic Treg differentiation through epigenetic modifications in the *foxp3* locus, which induces FOXP3 expression and pTreg conversion (171, 174–176). *Clostridia* are known to produce high levels of butyrate and colonization of germ-free mice with these bacteria increased colonic Treg frequency and protected mice from colitis (177). By contrast, segmented filamentous bacteria (SFB) induced Th17 cell development in the gut promoting systemic autoimmunity (178–180). In a recent study, Luu et al. have shown that the SCFA pentanoate inhibited SFB-promoted Th17 cell induction by metabolic and epigenetically reprogramming $CD4^+$ T cells to suppress IL-17 production and fostering IL-10 production in $CD4^+$ T cells and B cells (181). Furthermore, Häger et al. reported increased Treg numbers in 36 RA patients after receiving high-fiber dietary supplementation for 28 days, which correlated with a higher Th1/Th17 ratio and decreased expression of markers associated with bone erosion (182).

Several studies have shown that administration of probiotic bacteria containing members of the *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* genera primed DCs to induce the development of $FOXP3^+$ Tregs and IL-10-secreting regulatory T cells (183, 184) (**Figure 1**). Poutahidis et al. showed that mice fed with Westernized "fast food"-style chow developed obesity and had increased IL-17 levels. By contrast, the addition of probiotic yogurt containing *Lactobacillus reuteri* into the diet was sufficient to induce weight loss by a Treg dependent mechanism (174). Importantly, diet alters the gut microbiome (185–187) and dysregulation of intestinal microbiota is associated with autoimmunity [reviewed in (188, 189)]. Wilck et al. have shown that increased salt consumption affects intestinal bacterial composition in mice and humans. *Lactobacillus* spp. was suppressed in high salt condition, but its supplementation prevented high salt-induced Th17 differentiation and ameliorated salt-sensitive hypertension and EAE severity (37, 154). Cekanaviciute et al. found that MS patients have a high presence of the *Akkermansia calcoaceticus* and *Akkermansia muciniphila,* and the exposure of healthy donor PBMC to these bacteria impaired Treg conversion while enhancing Th1 differentiation (190). These data connect diet with microbiota composition and autoimmune pathogenesis, raising the potential of microbiota-targeted therapies.

DIET AS A THERAPEUTIC AID TO CONTROL AUTOIMMUNITY

It is becoming clear that nutrition, metabolic state, microbiota, and autoimmunity are deeply interconnected. In addition to genetic factors, the Western diet characterized by high caloric intake in the form of processed food enriched in protein, sugar, fat and salt, is widely believed to contribute to the rise in autoimmune diseases in the last decades (**Figure 1**). However,

one of the major challenges in investigating the effect of diet in human health is the impossibility to address the role of individual nutrients, which maybe the reason why a definite association between dietary interventions and outcomes in human autoimmune disease has not been established yet. Besides, dietary nutrients and microbial metabolites alter the immune response by acting on different immune cell populations, challenging our aim to identify underlying immunological mechanisms targeted during dietary interventions. For instance, we have recently corroborated that high salt diet lead to alterations in T cell populations in murine tumor transplantation models (191). However, inhibition of tumor growth given by high salt diet was largely independent of T cells in these models. Instead, high salt blocked the suppressive function of myeloid derived suppressor cells (MDSCs) *in vitro* and seems to promote thereby more pronounced anti-tumor immunity *in vivo* (191).

Obesity alters the balance between pro-inflammatory and suppressive T cells responses in adipose tissue, with Tregs losing their phenotypic identity and function (116), and resulting in break of self-tolerance (131) (**Figure 1**). Caloric restriction exerts immunoregulatory effects but is not suitable as general therapy for humans. Interestingly, Cignarella et al. have recently reported that intermittent fasting also improves disease outcomes in the EAE model as caloric restriction does (192). This effect was partially mediated by changes in the gut microbiota, since microbiota transplantation from mice under intermittent fasting into normally-fed mice could induce protection from EAE (192). Microbiota is a major determinant in the regulation of pro-inflammatory and regulatory T cell plasticity in the gut (35–37). Importantly, gut-resident T cells have the ability to traffic between different organs and exert a systemic effect in the organism (193, 194). Furthermore, these findings were translated into a small trial studying 16 MS patients that were on intermittent fasting for 15 days. Although no significant changes in gut bacteria composition was observed, a trend toward increased abundance of the Treg-inducer *Clostridia* bacteria was reported (177, 192).

As indicated by these data, dietary interventions and the use of probiotics may aid in the control of Treg stability and function by altering the milieu in which Tregs act *in vivo*, and help to restore immune responses in individuals with autoimmune prone Western lifestyle.

CONCLUDING REMARKS

Although it is clear that Treg function is frequently altered in human autoimmunity, it should be noted that Tregs are a heterogenous population with distinct tissue-specific features, multiple functions and differential degree of plasticity in response to environmental cues. Moreover, autoimmune diseases are highly heterogenous and it is likely that different defects in Treg-mediated regulation are involved in different types of autoimmune disease and even in each individual depending on the specific genetic background (195). Increasing progress in purifying and subdividing Treg subsets and defining the mechanisms that dictate their function and plasticity will likely contribute to a better understanding on the role of Tregs in autoimmunity.

Dietary factors, via direct effects on immune cells or by acting indirectly through modulation of the gut microbiota, may regulate Treg plasticity and function and, therefore, may have the potential to control disease outcome. However, more research and tightly controlled studies are needed to assess the impact of specific dietary nutrients and bacteria or microbial metabolites on Tregs, autoimmunity, and human health.

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Regulatory T Cells Beyond Autoimmunity: From Pregnancy to Cancer and Cardiovascular Disease

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The evolution of the full range of functions of regulatory T cells (Treg) coincides with the evolution of mammalian pregnancy. Accordingly, Treg function has been shown to be crucial for maternal-fetal tolerance and implantation. As reproduction is a key point of selective pressure, mammalian pregnancy may represent an evolutionary driver for the development of Treg. Yet beyond the chronological boundaries of mammalian pregnancy, several key physiological and pathological events are being gradually uncovered as involving the immunomodulating functions of Treg cells. These include autoimmunity, age-related inflammation in males and in post-menopausal females, but also oncological and cardiovascular diseases. The latter two sets of diseases collectively compose the main causes of mortality world-wide. Emerging data point to Treg-modulable effects in these diseases, in a departure from the relatively narrower perceived role of Treg as master regulators of autoimmunity. Yet recent evidence also suggests that changes in intestinal microbiota can affect the above pathological conditions. This is likely due to the finding that, whilst the presence and maintenance of intestinal microbiota requires active immune tolerance, mediated by Treg, the existence of microbiota *per se* profoundly affects the polarization, stability, and balance of pro- and anti-inflammatory T cell populations, including Treg and induced Treg cells. The study of these "novel," but possibly highly relevant from an ontogenesis perspective, facets of Treg function may hold great potential for our understanding of the mechanisms underlying human disease.

Keywords: Treg, pregnancy, autoimmunity, cancer, cardiovascular disease, microbiota, evolution

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INTRODUCTION: SELECTIVE PRESSURE SHAPES FUNCTION IN Treg

Biological systems develop as serendipitous solutions to selective pressure, according to evolutionary theory. The evolution of regulatory T (Treg) cells and their master regulator transcription factor, *foxp3* (1), must have occurred in response to selective pressure that conferred an advantage to vertebrates that possessed them. Whilst an early form of *foxp3* does exist in zebrafish (2), the full set of domains of *foxp3* only appear in the non-placental mammal platypus (3). Additionally, the enhancer element that is necessary for the induction of induced Treg (iTreg) in the periphery, also first appears in the platypus (4).

The platypus is an egg-laying mammal, and the egg creates a barrier separating the (non-self) paternal antigens from the maternal adaptive immune system. Absence of a barrier would necessitate a mechanism of suppression of maternal anti-fetal responses, a requirement termed "immunological paradox of pregnancy" by transplantation pioneer Medawar (5).

On the other hand, all subsequent (in terms of speciation) mammals are placental, having dispensed with the egg, benefiting from the advantage of a continuous flow of nutrients to the fetus. Thus, one can speculate that the serendipitous acquisition of an immunosuppressive T cell subpopulation could have enabled the elimination of the egg barrier.

In support of such a speculation we and others have shown that placental pregnancy with a genetically different father is not possible in the absence of regulatory T cells (6–8). Defects in Treg cells are associated with increased early-stage miscarriage and preeclampsia in humans (9, 10). In summary, whilst a robust adaptive immune system, as developed in vertebrates, is essential in maintaining defense of the self against pathogens (11), the evolution of Treg cells in placental mammals may have enabled the more complex management of the distinction between the self vs. the "non-self of the same species". The recognition of non-self of the same species, which is central in placental pregnancy, is ironically a much older problem, as sea-dwelling protochordate *Botryllus* had to fend off -and not tolerate- competition from neighboring individuals of the same species, using molecular processes not too dissimilar from those of Natural Killer (NK) cells (12). In mammalian pregnancy, maternal uterine NK cells interacting with non-classical Class I Major Histocompatibility molecules, such as HLA-G, independent of presence or absence of alloantigen, are essential for vascularization of the placenta, especially at the start of pregnancy (13, 14).

Treg IN PREGNANCY: A FLUCTUATING BUT REGULATED POPULATION

Evidence from mice and humans demonstrates that the abundance of Treg cells is modified during events linked to placental pregnancy. Periodic fluctuations in uterine (15) or peripheral (16) Treg levels render the cells more abundant during the fertile window of the estrus/menstrual cycle, so that suppression can take place should a pregnancy occur. These fluctuations are possibly estrogen-driven, as estrogen has been shown to boost Treg function (17, 18), whilst estrogen-depleting ovariectomy reduces Treg cell abundance (19). Once fertilization occurs, a much more substantial expansion of Treg cells can be observed (6). In this expansion, a role for paternal and male antigen-driven expansion of Treg has been demonstrated; initially in response to seminal fluid antigens (20), as well as paternal antigens (8, 21), which may explain the clonal expansion of Treg cells in the decidua but not the periphery in pregnant women (10).

Intriguingly, the pregnancy-associated expansion can be interrupted, should a uterine infection appear that could jeopardize fetus and mother (22). From a speculative evolutionary perspective, pathogen-induced reductions in Treg functionality would be selected for, as they would spare the mother from pathogens that could expand uncontrollably in an immunosuppressed environment. A putative mechanism may involve recognition of the pathogen by IL-6-producing innate immune cells, blocking the suppressive potential of Treg (23). Indeed, IL-6 is associated with fertility and pregnancy-related pathologies (24), and the cytokine is also known to mediate a conversion of Treg cells into Th17 pro-inflammatory cells in autoimmune arthritis (25). It should be noted that danger signal-induced fetal rejection can be mediated by invariant/semi-invariant lymphocytes, such as iNKT cells (26), Mucosal-Associated Invariant T cells (27) or γδ T cells (28).

Treg IN AUTOIMMUNITY

It is reasonable to ask how the function most often ascribed to Treg cells, the control of autoimmunity, fits with their role in placental reproduction. Pregnancy is known to temporarily alleviate the symptoms of rheumatoid arthritis in a majority of patients (29). In a murine model of autoimmune arthritis we have shown that the pregnancy-driven expansion of Treg is indeed responsible for this amelioration (30).

A few studies on peripheral blood Treg levels in human patients have shown a maintenance of their elevated numbers in the short period post-partum (31). Nonetheless, in antigen-specific murine models, which can be monitored with more precision, long-term increases in Treg levels from previous pregnancies were much lower than the peak reached during pregnancy (8). Consequently, Treg levels must be dropping after every pregnancy. The same could occur at menopause, as the ovariectomy-induced Treg contraction suggests (19). The time period post-partum and menopause are the main windows of incidence of most -though not all (32)—autoimmune diseases, which often affect far more women than men. In a logical corollary of an evolutionary selection-driven role for Treg in pregnancy, this higher incidence of autoimmunity in women may be an unwanted consequence of an estrogen-responsive Treg population that is necessary for an improved, placental reproduction. Such unwanted deleterious effects of selected-for earlier benefits fall within the term antagonistic pleiotropy (**Figure 1**), often applied to describe the benefit of infection-fighting immunity in young age leading to deleterious inflammation in old age (33). As pregnancy is the timepoint of genetic heredity, conditions after pregnancy may potentially feature antagonistic pleiotropy effects.

AFTER MENOPAUSE AND IN MEN: AGE-RELATED DISEASES

The absence of estrogen fluctuation in ovariectomized female mice induces not only a reduction in Treg levels but also a shortening of their lifespan, which approaches that of male mice (19). This is compatible with an overall beneficial effect of Treg in females undergoing estrus/menstrual cycles. If this conjecture were to be true, it would lead to a propensity for inflammation-associated disease in post-menopause women and in all men. Murine models may not be the optimal subjects of study for this question, as mice do not undergo menopause; only humans and cetaceans do (34), possibly as a result of socially-driven selection. Further, modern humans have another unique

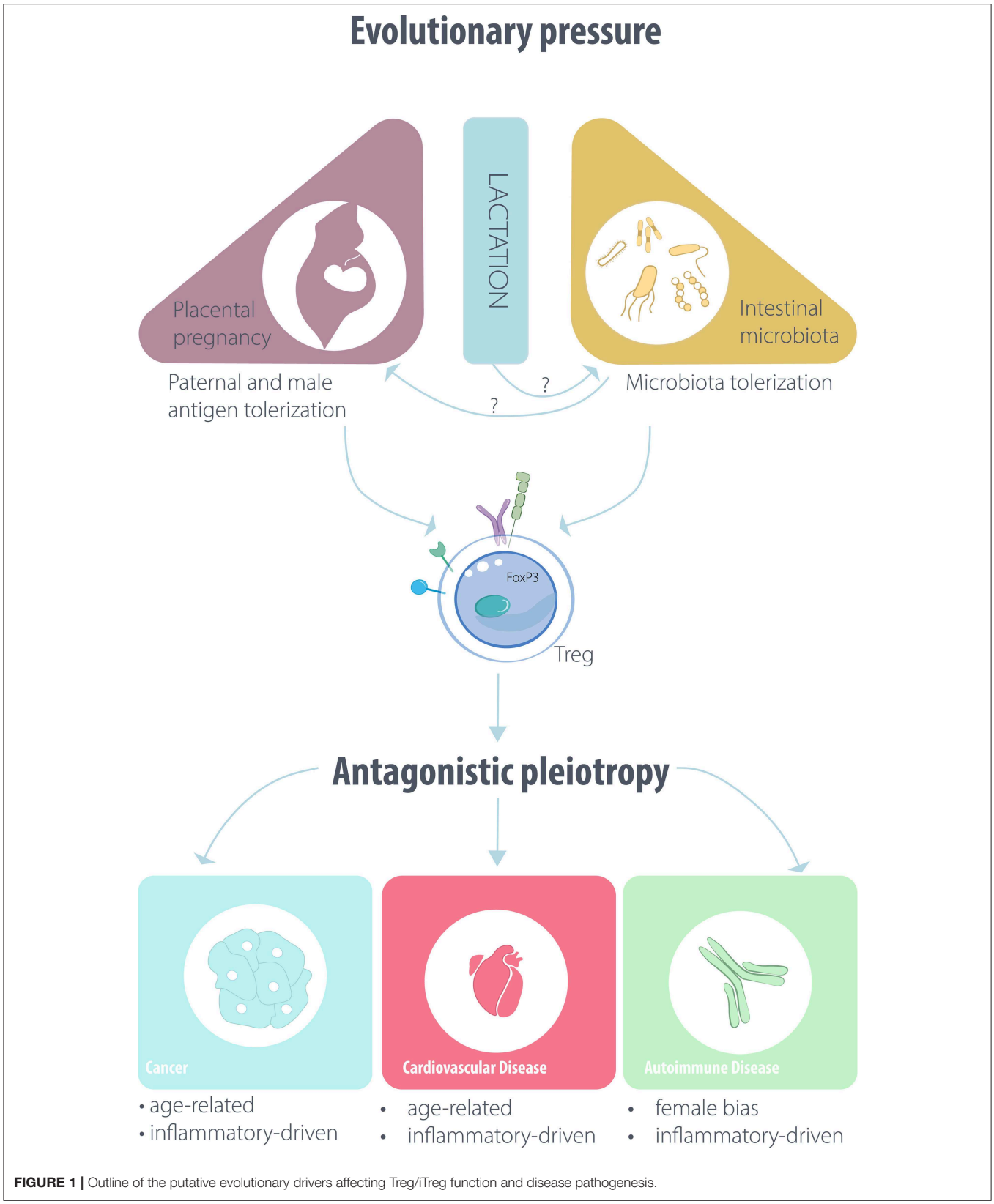

FIGURE 1 | Outline of the putative evolutionary drivers affecting Treg/iTreg function and disease pathogenesis.

feature: compared to other primates or even human hunter-gatherer populations, modern humans live substantially longer, with longevity data forming a branching point corresponding to the first industrialized societies (circa 1850), when sanitation was applied in a large scale (35). We can therefore hypothesize that if Treg function has been selected, in all mammals from the platypus onwards, thanks to its beneficial effects in enabling pregnancy, it would still nonetheless be involved, beneficially or detrimentally, in the regulation of any inflammation-associated ailment that occurs in our recently-acquired long lifespans. The reported decay of thymic Treg and iTreg with advanced age (36) could indeed contribute to a loss of self-tolerance to antigens expressed by aging tissue, promoting inflammation-associated disease pathogenesis. The ailments that predominantly affect older humans and lead to the majority of human mortality in the developed world are cancer and cardiovascular disease (37). Fitting our conjecture above on a Treg-related benefit prior to menopause, cardiovascular disease has higher incidence in men than in women, with a difference that decreases with older age (38). For all these reasons, thus, we would expect to find regulatory roles for Treg cells within these groups of diseases.

Treg AND CANCER

The link between inflammation and cancer is two-pronged. On the one hand, extensive findings have demonstrated that pro-inflammatory cytokines may enhance the chances of carcinogenesis and genetic instability (39). Treg-mediated suppression of such oncogenic inflammation would be beneficial. Such events clearly happen away from the clinically observable conditions of cancer patients, whose diagnosis occurs long after the carcinogenic event; this may be limiting the incentive to study the role of Treg cells in carcinogenesis. Yet even in growing tumors, evidence has shown that formation of tumor-promoting fibrotic capsules around prostate tumors occurs only in the presence of pro-inflammatory T cells (40), selective suppression of which would be beneficial.

On the other hand, the most clinically important interaction between immunity and cancer is the anti-tumoral, pro-inflammatory function of immunosurveillance (41), which has enabled the development of tumor immunotherapy. The latter, in its most applicable form of immune checkpoint blockade immunotherapy, is based on antibody-mediated reactivation of pro-inflammatory T cells. Yet Treg cells express and utilize the immunotherapy target molecules CTLA-4 (42) and PD-1 (43), and the suppressive action of the Treg may be inhibiting beneficial anti-tumor immunity (44). Why would Treg cells inhibit an anti-tumor response? Interpreted according to the signals a Treg cell may have evolved to deal with, a tumor expressing self-antigens and neo-antigens may be not that different from a fetus, the putative driver of the Treg cells' selection. Genes and processes that help fight non-pediatric, growing, solid tumors cannot have been inherited and selected for in mammals, as until very recently it was not possible to survive and reproduce following cancer incidence.

And yet an obvious solution does arise from the, admittedly speculative, study of the evolutionary drivers of Treg function. As hypothesized above, Treg suppression could collapse in order to reject an infected fetus, in order to protect the mother from the infecting pathogen. In this context, as pioneered in principle by Coley's toxin (45), vaccination strategies that fool the immune system into identifying the tumor as an infected fetus may represent tools that are aligned with the evolutionary drivers of the biological components that we are trying to modulate (46).

Treg AND CARDIOVASCULAR DISEASE

Cardiovascular diseases, ranging from atherosclerosis to myocardial infarction (MI) and heart failure, are not traditionally thought of as linked to immunity. Over the recent years, experiments showing that stressed cardiomyocytes release pro-inflammatory cytokines (47) led to clinical trials aiming to therapeutically inhibit the cytokine activity via monoclonal antibodies. As these failed (48), renewed efforts centered on identifying the adaptive immune cells involved in the progression of pathogenesis in atherosclerosis and heart failure. This was based on the premise that a chronic immune response may well be under the control of adaptive immunity. Accordingly, a role for Treg was identified in atherosclerosis (49), whilst therapeutic effects by the experimental administration of Treg cells in a model of pressure overload induced heart failure were also reported (50). In a more translational approach, we have used a molecule derived from Treg, CTLA-4, in soluble fusion protein form (CTLA-4-Ig/Abatacept) to treat advanced-stage heart failure in the pressure overload model. Surprisingly, the treatment with the drug, which is FDA-approved for use in Rheumatoid Arthritis patients, was almost 3-fold more effective than the current standard therapy for heart failure, demonstrating the potential of Treg-inspired therapeutic strategies (51).

More recently we identified, via single cell RNA sequencing, that Treg cells found to be infiltrating the ailing myocardium, express PD-1. Inhibition of PD-1 in healthy hearts blocked the Treg-mediated suppression, releasing cardiac inflammation, which in turn led to a significant reduction in heart function (52). This is intriguing, as anti-PD-1 treatment in human cancer patients has been shown to occasionally lead to T cell-mediated fulminant myocarditis (53). Luckily a solution exists, as CTLA-4-Ig treatment of tumor immunotherapy-induced myocarditis patients has a rescuing effect (54).

In MI that progresses to chronic ischemic heart failure, very recent evidence suggests that Treg cells may lose their immunosuppressive properties, becoming pro-inflammatory and worsening disease outcome (55). Their role is somewhat less clear in the early phase of post-MI repair, where the pro-inflammatory conventional T cells may be useful in the short term in order to deal with the extensive tissue damage (56, 57).

Treg AND INFECTIONS

Treg can dampen the response against pathogens during an infection, limiting collateral damage. As a consequence, this also leads to pathogen persistence, which in turn

boosts the persistence of protective immunity against the pathogen itself (58). Yet, simultaneously, the inflammation associated with the response limits the functionality of Treg cells (59), a finding that matches the inhibition of Treg function induced by danger signals, mentioned above (23), or indeed by inflammation *per se*, including in contexts of cardiovascular disease (60).

Treg AND INTESTINAL MICROBIOTA

The fetus expressing paternal antigens is not the only "non-self" that our adaptive immune system has to tolerate via Treg cells. Intestinal microbiota are essential for our survival and are not rejected (61), despite reaching very high cell numbers in the gut (62). The tolerization of "useful" bacteria may be mediated via Treg-mediated suppression (63), whereas "harmful" bacteria may be attacked by pro-inflammatory T cell subpopulations (64). Conversely, both anti-inflammatory iTreg and pro-inflammatory Th17 cells are induced in the gut, displaying a plasticity that depends on the microbiota (65, 66). For example, the immunomodulatory capsule polyaccharide A (PSA) of *Bacteroidetes fragilis* has been shown to induce IL-10-secreting Treg cells in the gut, restraining gut inflammation (67). Further, bacterial metabolites such as short-chain fatty acids (SCFAs), are involved in Treg differentiation (68–70).

Consequences of the microbiota-induced plasticity may affect disease pathogenesis. The anti-tumoral, pro-inflammatory effect of anti-CTLA-4 or anti-PD-1, described above, was abolished in experimental systems where the intestinal microbiota were eliminated (71, 72), demonstrating the potency of the microbiota-mediated effects. In agreement with these striking results, multiple translational studies have now highlighted how the microbiome of patients that respond to anti-PD-1 treatment is significantly different from that of non-responders (73), and how antibiotic treatment in combination with anti-PD-1/anti-PD-L1 immunotherapy can have a direct effect on patient survival rate (74).

In an analogous manner, emerging evidence demonstrates that the microbiome can significantly affect the pathogenesis and outcome of cardiovascular disease. Alteration of the gut microbiota has been associated with atherosclerotic lesion formation, as revealed by gut metagenome analysis in patients (75). Gut microbiota-produced SCFAs have even been shown to affect blood pressure regulation (76). The above findings exemplify how gut microbiota, possibly also via their effects on iTreg/Th17 populations, have substantial, though still largely unexplored, regulatory roles on the major disease groups that drive mortality world-wide.

CONCLUDING REMARKS—AN EVOLUTIONARY ROLE FOR MAMMALIAN GUT FLORA UPSTREAM OF Treg AND PLACENTAL PREGNANCY?

The hypothesis that pregnancy may have been a driver for the selection and survival of estrogen-responsive, immunosuppressive Treg cells that enable us to reproduce via a placenta and not using eggs is attractive. The enhanced ability to provide a continuous flow of nutrients to the fetus could even be hypothesized to have helped select bigger brains, capable of abstract thought. However, the interaction between Treg cells and the intestinal microbiota, which is central to our physiology and pathology, raises an intriguing possibility. The evolution of fully-functional *foxp3* may have indeed been a prerequisite for the evolution of placental mammalian pregnancy. At least until further species are sequenced, the full set of *foxp3* domains appears to have first appeared in the platypus. Yet Treg evolution in the platypus cannot have had pregnancy as a driver of selective pressure, as the platypus is lactating but does not have a placenta. We could, however, hypothesize that, prior to pregnancy, the intestinal microbiota required to digest milk in mammals, which first appeared in the platypus, offering clear advantages as a source of readily-available calories for the litter, could be distinctly different from those found in non-mammalian vertebrates. Could such a bacterial diversification drive the selection of a dedicated tolerizing immune cell population? Could this immunosuppressive population only then enable the evolution of pregnancy in the next speciation step? Compatible with this hypothetical conjecture, the intestinal microbiota of lactating animals has been recently found to be significantly different (77). Further experimentation will be required to assess the validity of these hypotheses.

The interaction between intestinal microbiota and Treg, especially if the conjecture that the former may have been a driver for the evolution of the latter is valid, offers novel means of investigating the functional aspects of Treg cells. In the long term, one would hope that this will lead to innovative therapeutic strategies, in the context of autoimmunity, cancer and cardiovascular disease.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Human Regulatory T Cells From Umbilical Cord Blood Display Increased Repertoire Diversity and Lineage Stability Relative to Adult Peripheral Blood

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The human T lymphocyte compartment is highly dynamic over the course of a lifetime. Of the many changes, perhaps most notable is the transition from a predominantly naïve T cell state at birth to the acquisition of antigen-experienced memory and effector subsets following environmental exposures. These phenotypic changes, including the induction of T cell exhaustion and senescence, have the potential to negatively impact efficacy of adoptive T cell therapies (ACT). When considering ACT with $CD4^+CD25^+CD127^{-/lo}$ regulatory T cells (Tregs) for the induction of immune tolerance, we previously reported *ex vivo* expanded umbilical cord blood (CB) Tregs remained more naïve, suppressed responder T cells equivalently, and exhibited a more diverse T cell receptor (TCR) repertoire compared to expanded adult peripheral blood (APB) Tregs. Herein, we hypothesized that upon further characterization, we would observe increased lineage heterogeneity and phenotypic diversity in APB Tregs that might negatively impact lineage stability, engraftment capacity, and the potential for Tregs to home to sites of tissue inflammation following ACT. We compared the phenotypic profiles of human Tregs isolated from CB versus the more traditional source, APB. We conducted analysis of fresh and *ex vivo* expanded Treg subsets at both the single cell (scRNA-seq and flow cytometry) and bulk (microarray and cytokine profiling) levels. Single cell transcriptional profiles of pre-expansion APB Tregs highlighted a cluster of cells that showed increased expression of genes associated with effector and pro-inflammatory phenotypes (*CCL5*, *GZMK, CXCR3, LYAR*, and NKG7) with low expression of Treg markers (*FOXP3* and *IKZF2*). CB Tregs were more diverse in TCR repertoire and

homogenous in phenotype, and contained fewer effector-like cells in contrast with APB Tregs. Interestingly, expression of canonical Treg markers, such as FOXP3, TIGIT, and IKZF2, were increased in CB $CD4^+CD127^+$ conventional T cells (Tconv) compared to APB Tconv, post-expansion, implying perinatal T cells may adopt a default regulatory program. Collectively, these data identify surface markers (namely CXCR3) that could be depleted to improve purity and stability of APB Tregs, and support the use of expanded CB Tregs as a potentially optimal ACT modality for the treatment of autoimmune and inflammatory diseases.

Keywords: cord blood, peripheral blood, regulatory T cells, Tregs, adoptive cell therapy, scRNA-seq, immunosequencing

INTRODUCTION

The human immune system undergoes dramatic changes over the course of a lifetime in order to maintain tissue and organism homeostasis. Highly variable cellular dynamics abound during growth and development in early life. This is particularly apparent during the nascent perinatal period, as the periphery is actively seeded with innate and adaptive immune cells that quickly gain initial exposures to foreign antigens (1). These early priming events must confer protection from pathogens, while also maintaining peripheral tolerance to microbial commensals, inert environmental antigens, and self-tissues. Interestingly, despite inconsistent immune profiles in umbilical cord blood (CB) and newborns, a recent report suggests individuals eventually converge on a common post-natal trajectory for healthy/normal immunological development (1). Efforts to define this common trajectory and the disruptions that give way to immune-mediated diseases represent an essential line of investigation. A growing body of evidence supports the notion that a breakdown in the establishment of peripheral immune tolerance is at the heart of many inflammatory and autoimmune disorders [reviewed in (2)]. While many cell types contribute to immune homeostasis, it is now appreciated that a unique subset of thymic regulatory T cells (tTreg) plays a vital role in establishing and maintaining dominant tolerance to self-antigens in the periphery (3). In fact, tTregs are so essential for maintaining immune homeostasis that loss-of-function mutations in FOXP3, the canonical transcription factor that marks the Treg cell lineage, can result in the lethal multi-organ autoimmune disease referred to as immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome [reviewed in (4)].

The identification of tTregs, and the subsequent development of methods for their *ex vivo* isolation and expansion from peripheral blood, has led to an explosion of research interest to harness these cells to control autoimmune diseases, inflammatory disorders, and enable tissue engraftment in the context of transplantation (5–8). The application of *ex vivo* expanded cells to achieve clinical outcomes is broadly referred to as adoptive cell therapy (ACT). ACT with T cells has advanced largely from pioneering work in the cancer immunotherapy space with the goal of tumor-directed immunity (9–15). These endeavors have identified critical factors determining robust clinical response and efficacy. While not comprehensive, these include key parameters of antigen-specificity of the therapeutic T cells (i.e., either polyclonal or antigen-specific) (16–18); lineage stability of the population that is used for ACT (19, 20); and the capacity of the T cells to traffic to proper sites *in vivo*, engraft into tissue microenvironments, and exert their context-dependent effector functions (21, 22). While the desired functions of Tregs in restoring immune regulation contrast those of effector T cells (Teff) targeting cancer, the core concepts governing specificity, stability, and functional capacity are likely to be highly analogous for the use of Tregs to treat autoimmune diseases, including type 1 diabetes (T1D).

Translating these early advances into efficacious therapies with Tregs is likely to require a more robust understanding of Treg biology. Specifically, there is a need for a more complete knowledge of the phenotypic changes that occur over the course of a human lifespan. Murine studies have demonstrated that tTregs generated during the perinatal period display a distinct receptor repertoire and are functionally different from tTregs isolated from mature mice (23). Human adult peripheral blood (APB) Tregs are comprised of a complex mixture of resting and activated subsets (24) and are known to co-opt the transcriptional profiles of the various T helper (T_H) cell subsets they are tasked with suppressing (24–28). In addition, APB Tregs are reported to display lineage instability resulting in effector-like T cell phenotypes (29–31). However, the heterogeneity of Tregs in CB is generally uncharacterized.

Our prior work has demonstrated that tTregs can be isolated from human CB and expanded with exceptional purity and lineage stability (32). Here, we extend our prior studies optimizing Treg expansion protocols to further characterize the transcriptional profile and repertoire characteristics of human Tregs from CB in comparison to those isolated from APB. We employed both bulk transcriptional profiling, as well as single cell RNA sequencing (scRNA-seq) and T cell receptor (TCR) repertoire analyses to characterize CB and APB Treg populations that could be harnessed for ACT. Our novel transcriptional profiling data and repertoire analyses once again reinforce the concept of a phenotypically homogenous and lineage stable Treg population in CB when compared to APB. These studies have implications for identifying optimal cell sources for either autologous or allogeneic ACT applications. Moreover, the scRNA-seq data provide an array of novel cell

surface targets that can be leveraged to further optimize Treg isolation strategies for use in Treg ACTs for the induction of immunological tolerance.

MATERIALS AND METHODS

Sample Collection and Processing

Fresh CB (processed within 24 h of birth) was obtained from LifeSouth Community Blood Center Corporate Headquarters (Gainesville, FL) into CB units containing 35 mL of citrate phosphate dextrose anticoagulant. CB units ($n = 7$) were delivered to the University of Florida Diabetes Institute (UFDI) and immediately processed for CB mononuclear cells (CBMCs). Leukopaks containing fresh APB ($n = 6$) were purchased from LifeSouth Community Blood Center (Gainesville, FL, United States). These deidentified samples were obtained under an approved IRB exempt protocol at the UFDI. APB samples were processed within 24 h for isolation of peripheral blood mononuclear cells (PBMCs). For CBMC and PBMC isolation, CB and APB samples were subjected to $CD4^+$ enrichment with the RosetteSep® Human $CD4^+$ T Cell Enrichment Cocktail (STEMCELL Technologies) followed by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare) prior to fluorescence-activated cell sorting (FACS). The overall workflow for the experiments reported herein is summarized in **Figure 1**.

FACS of $CD4^+$ Tregs and Conventional T Cells (Tconv)

$CD4^+$ T cell enriched CBMCs and PBMCs were stained with fluorescently labeled antibodies, resuspended at 2×10^7 cells/mL, and sorted on a BD FACS Aria III Cell Sorter (BD Biosciences), as previously described (32). Tregs and Tconv were sorted as $CD4^+CD25^{hi}CD127^{lo}$ and $CD4^+CD127^+$, respectively.

T Cell Expansion

Tregs and Tconv from CB and APB were expanded as previously described (32). In brief, sorted Treg and Tconv were incubated with KT64/86 aAPCs at a 1:1 ratio in the presence of exogenous IL-2 and expanded for 14 days with restimulation using anti-CD3 anti-CD28 coated microbeads on day 9 following protocol 1 (32). Expanded CB Tregs, CB Tconv, APB Tregs, and APB Tconv were cryopreserved in CryoStor (Sigma, CS10) and later thawed for batched experiments as described below.

RNA Extraction and Quality Assessment

Following expansion, 3×10^5 CB Tregs, CB Tconv, APB Tregs, and APB Tconv were lysed in DNA/RNA lysis buffer (Zymo Research) and stored at −80°C. RNA extraction was achieved using ZR-Duet™ DNA/RNA MiniPrep (Zymo Research, Catalog No. D7001), per the manufacturer's instructions. Quality assessment of RNA was achieved by Experion™ Automated Electrophoresis System (BIO-RAD) using Experion RNA High Sensitivity Reagents and Experion Standard Sensitivity RNA chips following the manufacturer's protocol. Only samples with a minimum RNA concentration of 10 ng/μL and RNA Quality Index (RQI) ≥9.4 were used.

scRNA-seq and Library Construction

Gene expression and V(D)J libraries were prepared from 5,000 pre- and post-expansion CB and APB Treg cells using the Chromium Single Cell 5′ Bead and Library Kit v1 and the Chromium Single Cell V(D)J Human TCR Analysis Kit (10X Genomics). Libraries were sequenced on an Illumina HiSeq instrument at a target read depth of 50,000 reads per cell.

Processing of Sequencing Reads and Generation of Gene-Barcode Matrices

Raw sequencing reads were processed using Cell Ranger v3.0.0 to create a raw (unfiltered) gene-barcode matrix. Briefly, Cell Ranger mkfastq was used to make fastq files from bcl files. Next,

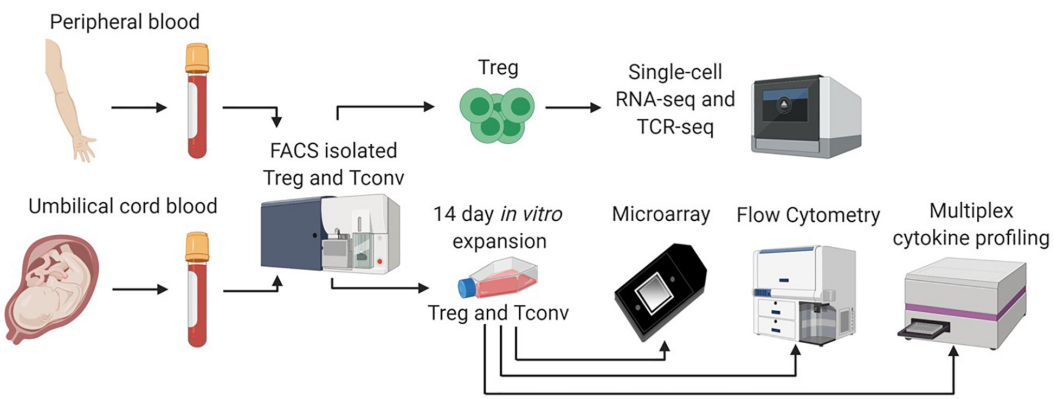

FIGURE 1 | Single cell and bulk sample analysis workflow. We adopted a multifaceted approach to assess differences between CB and APB derived Tregs. Fresh CB Tregs, CB Tconv, APB Tregs, and APB Tconv were fluorescence activated cell sorting (FACS) isolated. Sorted CB Tregs and APB Tregs were directly analyzed by single cell RNA sequencing (scRNA-seq) on the 10x Genomics platform. We assessed single cell gene expression and T cell receptor (TCR) repertoire differences. In addition, freshly sorted CB Tregs, CB Tconv, APB Tregs, and APB Tconv were expanded *in vitro* for 14 days, after which we performed scRNAseq, as well as bulk transcriptional analysis by microarray, flow cytometry and cytokine secretion analysis by Luminex assay.

Cell Ranger count was used for aligning sequencing reads to the hg19 reference genome (refdata-cellranger-hg19-3.0.0), obtained from[1] using STAR (33). For confidently mapped reads (as defined by Cell Ranger), Unique Molecular Identifier (UMI) sequences were collapsed and the number of UMI reads per gene were stored in the raw gene-barcode matrix[2].

Filtering of Barcodes/Quality Control and Normalization

Quality control was performed for scRNA-seq data from pre- and post-expansion APB and CB Tregs (**Supplementary Figure S1**). Barcodes associated with droplets containing cells were distinguished from ambient RNA droplets using the emptyDrops algorithm implemented in the DropletUtils R/Bioconductor package (34). Briefly, each barcode is tested for deviations from the estimated ambient RNA profile as defined by barcodes with 100 UMIs or less. Barcodes with a false discovery rate adjusted p-value < 0.01 were retained after this initial filter. A second filter based on the inflection point in the UMI rank versus total UMI curve was used for more stringent identification of cellular barcodes (**Supplementary Figure S1A**). Next, we filtered on commonly used quality control measures, such as the total number of UMIs per cell (library size), the number of genes expressed, and the percentage of mitochondrial reads per cell to identify cells with low RNA content, possible doublets, and presumably dead or damaged cells. Cells with a total UMI count or number of genes expressed greater than or less than three median absolute deviations (MADs) from the median were removed. Additionally, cells with a percentage of mitochondrial reads greater than three MADs from the median were removed. This filtering was implemented using the isOutlier function in the scran R/Bioconductor package (35) (**Supplementary Figures S1B,C**). Additionally, cells with more than one unique *TRB* chain and two unique *TRA* chains as defined by the concatenated V-gene, complementarity determining region 3 (*CDR3*) sequence, and J-gene were excluded as presumed doublets (**Supplementary Figure S1D**). 4320 cells from the APB and 4842 cells for the CB pre-expansion samples, and 4403 cells from post-expansion APB and 3842 cells from post-expansion CB Treg passed these filters and were used in downstream analyses. To remove variation in the number of molecules detected per cell, residuals from regularized negative binomial regression with library size as a covariate was used as described in (36) and implemented in the SCTransform function in Seurat v3.1 (37). Briefly, a negative binomial regression model is fit for each gene with the number of molecules per cell as a covariate and the read-count of the cell as the dependent variable. This method selects stable model parameters that are robust to overfitting by pooling parameter estimates across genes with similar abundances.

[1] https://support.10xgenomics.com/single-cell-gene-expression/software/release-notes/build

[2] https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/algorithms/overview

Dataset Integration and Dimensionality Reduction

The cord blood and adult peripheral blood datasets were integrated as detailed in (38) and implemented in Seurat. Briefly, canonical correlation analysis (CCA) was performed to identify shared sources of variation across the datasets, and mutual nearest neighbors in the CCA space were identified to produce anchors between datasets. For pre-expansion datasets, highly variable genes accounting for the majority of the heterogeneity within each sample were identified by ranking genes based on variance of the residuals from the regularized negative binomial regression model described above, again as described in (36) and implemented in the SCTransform function in the Seurat R package (37, 39). For post-expansion datasets, the corresponding variable features from the pre-expansion state were used, as the variable features post-expansion were dominated by cell-cycle driven expansion-related genes that were not of primary interest. Using these features, anchors between the datasets which correspond to similar cells across datasets were identified using the FindIntegrationAnchors function, and this was used as input into the IntegrateData function to generate an integrated dataset. For dimensionality reduction, expression values for each gene in the integrated dataset were scaled to have a mean of zero and standard deviation of one using the ScaleData function, and principal component analysis (PCA) was run on this matrix using the RunPCA function in Seurat (37, 39). For visualization, Uniform Manifold Approximation and Projection (UMAP), a common dimensionality reduction method in scRNA-seq, plots were created based on the top 20 principal components using the RunUMAP function in Seurat.

Clustering and Cluster Differential Expression Analysis

Cells were clustered into groups of similar transcriptomic profiles using graph-based clustering on the first 20 principal components of the integrated dataset. Briefly, a shared nearest neighbors graph was created based on the Jaccard similarity of the sets of the 20-nearest neighbors for each cell, as implemented in FindNeighbors function in Seurat (37, 39). Clusters were then identified by partitioning this graph using the Louvain community detection algorithm with a resolution of 0.4, as implemented in the FindClusters function in Seurat (37). Clusters sizes and the relationship between clusters at different resolutions were analyzed to determine this value (**Supplementary Figures S5, S6**) (40). DE genes across clusters were identified by comparing each individual cluster with the remaining pooled clusters for each sample using the Wilcoxon rank sum test implemented in the wilcoxauc function in the presto R package (41). *P*-values for each cluster from each sample were then combined using Wilkinson's method as implemented in the minimump function in the metap package (42) to identify conserved markers across datasets.

TCR Clonotype Assignment and Evenness Profile Calculation

Clonotypes were assigned to cells based on unique paired *TRA-TRB* V-gene/CDR3/J-gene sequences. Only cells with one β-chain

and one α-chain were assigned clonotypes to prevent artificial inflation of clone counts due to reduced information about the sequence. Evenness profiles were calculated as initially described (43). Briefly, for sample-level analysis, clonotypes were tabulated, and frequency vectors for each clonotype within a sample were calculated. Evenness profiles based on the exponential of Hill diversity were computed for α in the range 0–10, with step size 0.2, where ${}^{\alpha}E = \frac{{}^{\alpha}D}{{}^{\alpha=1}D}$ and ${}^{\alpha}D(\mathrm{f}) = \left(\sum_{i=1}^{n} \mathrm{f}_i^{\alpha}\right)^{\frac{1}{1-\alpha}}$, where ${}^{\alpha}E$ is the evenness for a given α and ${}^{\alpha}D$ is the Hill diversity for a given α, and f is the frequency vector. When $\alpha = 1$, while Hill diversity is not defined, it tends to Shannon entropy (43). This resulted in a 51-dimensional evenness profile for each sample. A large range of α was used to capture differences in clonal expansion across the clonal frequency distribution, as the majority of the clonotypes were single occurrence, and an increased α results in higher frequency clones being given a greater weight. The same procedure was followed for the cluster-level analysis, except each cluster from each sample is treated as an independent sample. Overall, the evenness profile is a low-dimensional vector containing the majority of the information contained in a clonal frequency distribution (44).

Microarray Studies

Post-expansion Treg transcript analysis was performed as previously described (45). Briefly, mRNA was reverse transcribed and amplified. Resulting cDNA was fragmented and labeled using the GeneChip WT Plus Kit and subsequently, hybridized onto the Clariom S Human Array (Thermo Scientific), following the manufacturer's procedures. Arrays were scanned with the GeneChip Scanner 3000 7G using AGCC software and subsequently normalized using RMA as implemented in Partek 6.6. GEO Accession #: GSE137301.

Differential Expression Analysis

The R/Bioconductor package limma (46) was used for differential expression of genes using a linear model using the lmFit function with a model matrix with no intercept and fixed effect for treatment (e.g., CB Treg, APB Treg, CB Tconv, and APB Tconv), blocking on donor, and specifying an inter-donor correlation using duplicateCorrelation, effectively treating donor as a random effect. Contrasts were specified using makeContrasts, and the contrasts were fit using contrasts.fit. Moderated t-statistics were then computed using the empirical Bayes moderation as implemented in the eBayes function.

Absolute Telomere Length Assay

APB and CB Treg DNA was assayed using the Absolute Human Telomere Length Quantification qPCR Assay Kit (ScienCell) according to the manufacturer's instructions, with the exception of the qPCR master mix, for which we used Syber Select Master Mix (Applied Biosystems). Briefly, DNA were isolated using the DNEasy Blood and Tissue Kit (Qiagen), quantified using a Qubit Fluorometer (Thermo Fisher), after which 5 ng was input into the assay per subject. Data were acquired on a Roche LightCycler480 instrument, exported into Excel and analyzed in GraphPad PRISM v8.

Flow Cytometry

Expanded cryopreserved Tregs and Tconv from CB and APB were thawed in RPMI complete media and stimulated with phorbol myristate acetate (PMA; 10 ng/mL) and ionomycin (500 nM) for 4 h at 37°C with the addition of Golgistop (BD Biosciences; 0.66 μl/mL). Cells were stained for surface and intracellular markers to assess differentiation and effector markers, chemokine receptors and activation status (**Supplementary Table S1**). Data were collected on an LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc). For each marker, the percentage of cells positive for the marker was modeled with a mixed effects model using the lmer function in the lme4 package (47) with treatment (e.g., CB Treg, APB Treg, CB Tconv, and APB Tconv) as a fixed effect and donor as a random effect. Pairwise contrasts for treatment were computed using the emmeans function in the emmeans package (48). For supplementary experiments, expanded cryopreserved APB and CB Tregs or CBMC and PBMC were thawed in RPMI complete media and restimulated with αCD3/28-coated microbeads at a 1:1 ratio (milltenyi) or soluble αCD3 (2 μg/mL, clone HIT3a, BD Biosciences) and αCD28 (1 μg/mL, clone 28.2, BD Biosciences), respectively, for 48 h at 37°C with the addition of Golgistop (BD Biosciences; 0.66 μl/mL) for the last 4 h. Data were collected on a Cytek Aurora and analyzed as above with statistics computed and data plotted using Graphpad PRISM software v8, as indicated in figure legends.

Multiplexed Cytokine Detection

Isolated expanded CB Tregs, CB Tconv, APB Tregs, and APB Tconv were stimulated in a 96-well plate with PMA (10 ng/mL) and ionomycin (500 nM) for 4 h at 37° C. IL-2, IL-10, IL-12 (p40), IL-12 (p70), IL-19, IL-20, IL-22, IL-26, IL-27 (p28), IL-28A, IL-29, and IL-35 were detected in the supernatant using the Bio-Plex Pro Human Treg Cytokine 12-Plex Panel (Bio-RadR) according to the manufacturer's procedures with the following modification. Standards were diluted in standard Diluent HB as opposed to culture medium to generate a seven-point curve. For each cytokine, log10 (concentration) was modeled with a mixed effects model using the lmer function in the lme4 package (47) with treatment (e.g., CB Treg, APB Treg, CB Tconv, and APB Tconv) as a fixed effect and donor as a random effect. Pairwise contrasts for treatment were computed using the emmeans function in the emmeans package (48).

Data Visualization

Data were visualized using the following R packages: ggplot2 (49), ComplexHeatmap (50), scanalysis (51), ggexp (52), and clustree (40). Flow cytometry data were analyzed in FlowJo software (Tree Star, Inc.) and raw data were exported to GraphPad PRISM v8 or R for statistical analysis.

Code Availability

An R package with runner scripts to reproduce all analyses and figures in this manuscript are available at https://github.com/keshav-motwani/tregPaper (53).

RESULTS

scRNA-seq Identifies Contaminants in Pre-expanded Tregs

We sought to identify differences in the composition of native (i.e., unexpanded) CB and APB Tregs at the single cell level that might contribute to non-Treg contaminants in a post-expansion cell product for use in ACT. After identification of 4842 high quality cells in CB (n = 1 subject) and 4320 in APB (n = 1 subject), datasets were normalized for cell-specific biases related to sequencing depth using the residuals of regularized negative binomial regression as described in (36). To enable direct comparisons between APB and CB, the datasets were integrated by identifying anchors between similar cells across datasets (38). We performed graph-based clustering on the top 20 principal components (PCs) of the integrated data, identifying a total of 6 clusters which are overlaid on a reduced dimensional representation of the first 20 PCs using UMAP (54) (**Figure 2A**). From visual inspection of the first two UMAP components (i.e., UMAP1 and UMAP2; **Figure 2A**), cells in clusters C01-C05 are largely clumped together, but cluster C06 is more of an outlier

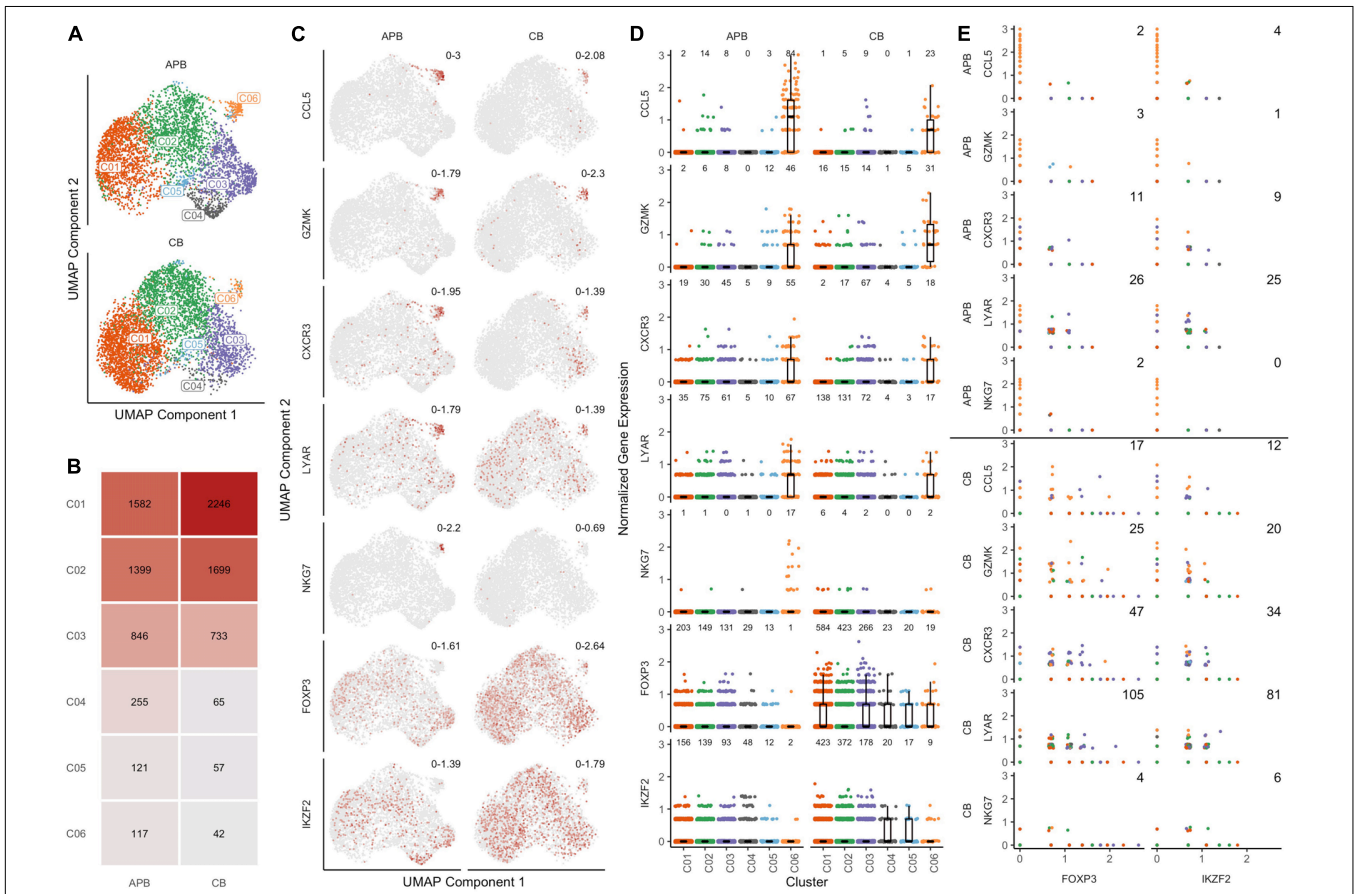

FIGURE 2 | Single cell gene expression profiles of unexpanded APB Tregs and unexpanded CB Tregs show the presence of a T_H1/T_H17-like contaminant cluster in APB. **(A)** UMAP plots for APB Tregs (n = 1, top) and CB Tregs (n = 1, bottom) colored by the assigned cluster number (C1-C6) shows the presence of a subpopulation (C06) that is not along the main trajectory and appears more prevalent in APB. **(B)** Absolute number of cells belonging to each cluster and relative abundances for each cluster within unexpanded APB Tregs (left) and unexpanded CB Tregs (right) show differences in cluster composition. Notably, C06 was comprised of 117 cells (3%) of APB Tregs versus 42 cells (1%) of CB Tregs. **(C)** For APB Tregs (left) and CB Tregs (right), UMAP plots are colored by expression of the five most differentially expressed genes in C06 (*CCL5*, *GZMK*, *CXCR3*, *LYAR,* and *NKG7*) as well as two canonical Treg genes (*FOXP3* and *IKZF2*). Numbers in the top right of each plot indicate the minimum and maximum expression for that sample. Points are colored based on the expression of the gene, with gray being the lowest value and dark red the highest value for that feature across both samples. **(D)** Visualizing expression of *CCL5*, *GZMK*, *CXCR3*, *LYAR*, *NKG7*, *FOXP3,* and *IKZF2* shows increases in "contaminant" gene expression in C06 and decreases in canonical Treg gene expression within this cluster from APB Tregs (left) and CB Tregs (right). The number of cells with nonzero expression of each feature in each cluster is annotated at the top of each plot. **(E)** For all clusters, pairwise scatterplots between canonical Treg genes *FOXP3* (left) and *IKZF2* (right) and the identified "contaminant" genes (*CCL5*, *GZMK*, *CXCR3*, *LYAR,* and *NKG7*) shows increased co-expression in CB Tregs (lower) compared to APB Tregs (upper). The annotated number represents the number of cells with nonzero expression for both genes. **(D,E)** For each data point, the color corresponds to the cluster number as presented in **(A)**.

(**Figure 2A**), and it is more pronounced in the APB sample (∼3% of cells in APB versus ∼1% in CB) (**Figure 2B**). To understand the underlying biology in each of the 6 subpopulations, we computed differentially expressed genes between each cluster and the rest of the cells in the dataset. For C06 in particular, the top five differentially expressed (DE) genes (ranked on *p*-value) were *CCL5*, *GZMK, CXCR3, LYAR*, and *NKG7* (**Supplementary Table S2**), as shown in the UMAP plots colored by relative expression (**Figure 2C**). *CXCR3*, *CCL5*, and *NKG7* have all been associated previously with T_H1 migratory capacity (55, 56) and phenotype (57), while the expression of *GZMK* and *LYAR* likely indicate a cytotoxic and activated population (58, 59). **Figure 2C** also depicts the canonical Treg markers, *FOXP3* and *IKZF2*, which were highly expressed in the majority of APB and CB Treg clusters but only lowly expressed in C06. **Figure 2D** shows the expression of these seven genes across all clusters where notably, there is a decrease in *FOXP3* and *IKZF2* expression and an increase in the C06 DE genes (*CCL5*, *GZMK, CXCR3, LYAR*, and *NKG7*). These trends are much more prominent in APB as compared to CB, due to the greater number of contaminant cells in APB. Moreover, there is greater co-expression of *FOXP3* and *IKZF2* with these five "contaminant cell" genes in CB compared to APB (**Figure 2E**). This could potentially indicate that contaminants expressing these T_H1-associated genes are present in both CB and APB, but the T_H1-like contaminants in CB still retain a Treg phenotype while in APB, they lose their regulatory phenotype and adopt an effector-like program. Further examination of the top 50 DE genes in C6 (**Supplementary Table S2**, ranked on combined *p*-value) shows upregulation of additional T_H1-associated genes including *BHLHE40* (60), *IFNG* (61), and *TBX21* (62); T_H17-related genes including *KLRB1*, which encodes CD161 (63), and *TGFB1* (64); as well as *IL12RB2* (65), shown to be expressed highly in $T_H1/17$ cells, which collectively suggests this contaminant population to belong to the recently characterized $T_H1/17$ subset (66). These data indicate that the CB Treg transcriptomic profile is more homogenous as a lineage as compared to APB Tregs, which contain non-Treg $T_H1/17$ contaminants with cytotoxic and pro-inflammatory potential.

CB Treg Repertoire Is Highly Diverse and Enriched in TCRs Associated With Self-Reactivity

Immune tolerance is initiated by tTregs that seed the periphery in early life, as reviewed previously (1, 23, 67). We and others have shown from bulk *TRB* immunosequencing that Tregs generally express a diverse repertoire of TCRs (32, 68–71). Here, we extended these studies to include paired *TRA* and *TRB* receptor analysis in unexpanded APB and CB Tregs. To understand how clonal expansion of cells related to their phenotype as represented on the UMAP plots, we compared expanded CDR3 sequences spanning the V and J genes for TCR-α and TCR-β chains (i.e., *TRAV*, *TRAJ*, *TRBV*, *TRBJ*) wherein every clone with a single occurrence was presented in gray while expanded clones with two or more occurrences were assigned a unique color (**Figure 3A**). Clonal expansion was observed in clusters 1, 2, 3, 4 (C01, C02, C03, and C04, respectively), and C06. To further characterize clonal expansion in each sample and the extent within each cluster we assessed receptor evenness profiles, which reflect the frequency vectors' distance from a uniform distribution and serve as a normalized diversity metric (**Figure 3B**) (43, 44). Overall, APB Tregs showed reduced diversity compared to CB Tregs (**Figure 3B**). This likely reflects TCR enrichments over time, presumably from chronic antigen exposures and selective pressures in the periphery. To determine if these differences were due to the influence of specific clusters, we compared the receptor evenness of each cluster between APB and CB (**Figure 3C**). Clusters 1 (C01) and 2 (C02) were moderately expanded in CB alone (**Figure 3C, red** and **green**), and were found to express genes related to Treg development, stability, and migratory capacity [*JUNB* (72), *DUSP2* (73), and *ITGB1* (74)], while the latter expressed genes associated with Treg activation and suppressive function [*IDI1* (75), *FCRL3* (76), and *ID3* (77)]. APB Treg cluster 3 (C03) demonstrated reduced receptor evenness in both APB and CB (**Figure 3C**, purple), and expressed genes related to T cell activation and memory phenotypes, namely *S100A4*, *S100A10*, *DUSP4*, *LGALS1*, and *LGALS3* (**Supplementary Table S2**) (78–81), as well as class II HLA and co-stimulatory molecules *TNFRSF4* and *TNFRSF18* (82, 83) and *PRDM1* (BLIMP-1) (**Supplementary Table S2**), likely representing a population of activated memory Tregs (84). C04 was also expanded in APB alone (**Figure 3C**, brown), and possessed an eTreg phenotype, with expression of *CCR4* (86) (**Supplementary Table S2**). Lastly, C06 (**Figure 3C**, orange), which is discussed extensively above, was expanded in CB, though the co-expression of T_H1-associated genes with *FOXP3* and *IKZF2*, which encodes Helios (**Figure 2**), suggests this population to potentially represent differentiated Tregs capable of suppressing the T_H1 effector lineage, as opposed to a T_H1 contaminant (87). Hence, among clusters exhibiting clonal expansion, the majority appeared to retain a regulatory identity with the exception of C06 within APB. Moreover, we were able to identify a cluster with a similar phenotype to C06 within expanded CB and APB Treg (C08, **Supplementary Figure S2**, and **Supplementary Table S3**), which expressed *KLRB1* and *IFNG*, as well as *CD40LG* (88), cytotoxic molecules *GZMA* and *GZMB* (58), and additional T_H17-associated genes *CCR6* (89) and *RORC* (90), indicating that the pre-expansion C06 cluster phenotype may still be relevant after expansion. Notably, we found post-expansion CB Treg to exhibit increased receptor evenness as compared to APB Treg (**Supplementary Figure S3**). Overlap between pre- and post-expansion contaminant cluster gene signatures is shown in **Supplementary Figure S4**. Additionally, both pre- and post-expansion, the contaminant cluster was a distinct cluster that was robust to changes in cluster resolution (**Supplementary Figures S5, S6**). Lastly, to understand the reactivities of the Tregs in APB and CB, we defined CDR3β sequence specificities using the manually curated catalog of pathology-associated T cell receptor sequences (McPAS-TCR) (91), matching the observed sequence composition (CDR3B) to sequences associated with putatively annotated reactivities and pathological conditions. Interestingly, we observed a greater

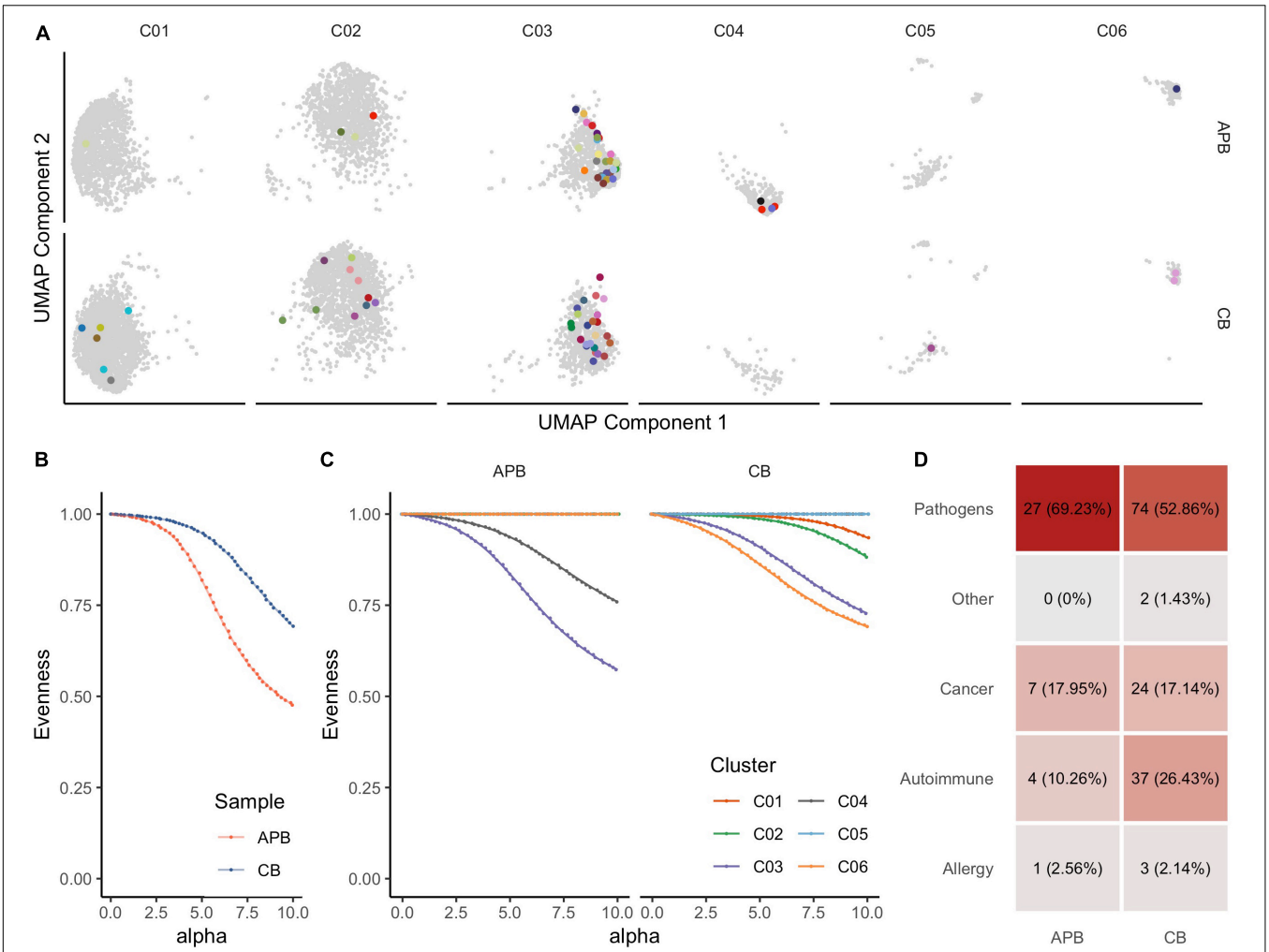

FIGURE 3 | T cell receptor (TCR) profiling from scRNA-seq data identifies clonally expanded subpopulations in pre-expanded Tregs. **(A)** UMAP plot colored by clonotypes with single occurrences (gray) and multiple occurrences (each clonotype distinctly colored) identifies clusters with expansion (UMAP Components 1 and 2 are the same as those presented in **Figure 2**). **(B)** Alpha value (i.e., clonotype frequency) versus evenness value (43) for APB Tregs (orange) and CB Tregs (blue) shows that along the range of the frequency distribution, APB Tregs show decreased evenness at all alpha values, thus exhibiting greater clonal expansion than CB Tregs. **(C)** When APB Tregs (n = 1, left) and CB Tregs (n = 1, right) are split by cluster (C01-C06, as defined in **Figure 2**), APB C03 and APB C04 contain expanded subpopulations, while CB C01, C02, C03, and C06 are also moderately expanded. **(D)** Putative antigen specificity composition of TCRs in each sample show an increased number of clones mapping to known reactivities, including autoimmune clones, in CB Tregs (right) compared to APB Tregs (left).

total number of CB Treg sequences corresponding to known predicted targets as compared to APB Tregs, and though there are comparable distributions of predicted reactivities, we observed an increased number of sequences with autoreactive specificity in CB Tregs (**Figure 3D** and **Supplementary Figure S4D**). These data suggest that even in the polyclonal state, CB Treg may be optimal for broad tissue engraftment with more clones expressing TCRs reactive to autoantigens when compared to Tregs derived from APB.

Expanded CB Treg Retain Lineage Stability and Phenotype

Achieving clinically effective Treg numbers in ACT often requires cell expansion (92). Moreover, it is essential that Tregs maintain a regulatory identity and the capacity for cycling and activation post-expansion. We previously determined CB Treg to maintain a naïve phenotype post-expansion (32) which is substantiated by our observation of increased telomere length in expanded CB Treg (CB mean: 383.4 ± 16 kb, APB mean: 258.2 ± 11 kb, **Supplementary Figure S7**). However, there is a paucity of data examining transcriptomic differences between expanded CB and APB Tregs. Therefore, we sought to address this by characterizing the transcriptome of CB and APB derived Tregs and Tconv by microarray after a 14 day *in vitro* expansion period. As expected (32, 93), among the top 30 DE genes between APB Treg and APB Tconv were the canonical Treg transcription factors *FOXP3* and *IKZF2*, along with various negative regulators and functional molecules, namely *TIGIT* and *TNFRSF9* (88), while APB

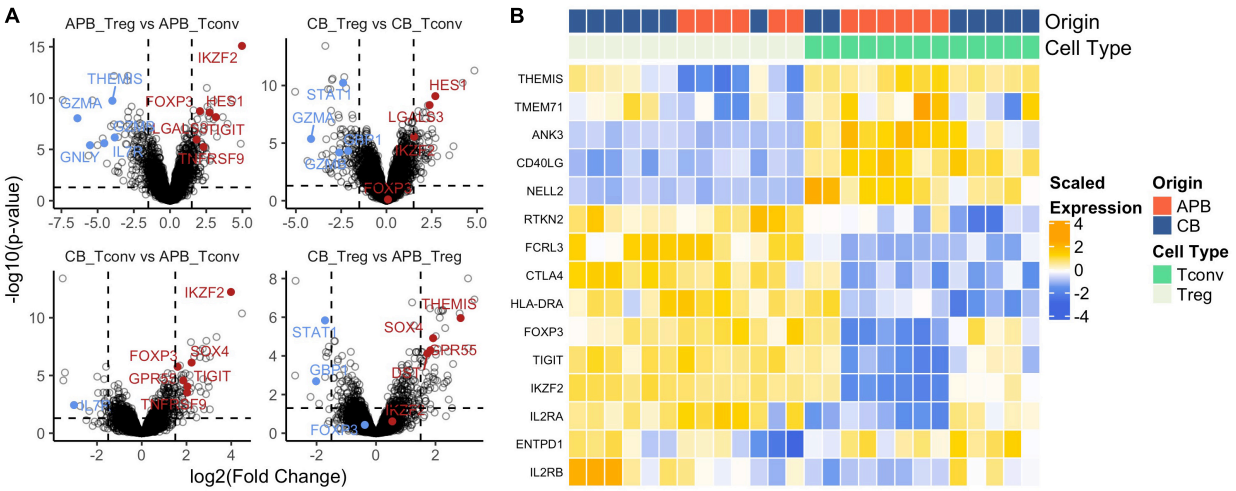

FIGURE 4 | Bulk gene-expression profiles from expanded cells show increased regulatory phenotype in both Tregs and Tconv from CB compared to APB. **(A)** Volcano plots showing log2 (Fold Change) versus -log10 (adjusted *p*-value) annotated with differentially expressed genes (155) colored by downregulation (blue) and upregulation (red). **(B)** Heatmap of canonical Treg and Tconv genes shows clustering by cell type (Treg (*n* = 13) versus Tconv (*n* = 13) and also sample origin (APB (*n* = 14) versus CB (*n* = 12).

Tconv preferentially expressed pro-inflammatory and cytotoxic mediators such as *GZMA*, *IL-7R*, *GZMB*, *GNLY*, and *IL18RAP* (**Figure 4A** and **Supplementary Table S4**). Differences in expression of canonical Treg genes were less robust between CB Tconv and CB Tregs (**Figure 4A** and **Supplementary Table S4**). Nevertheless, in CB Treg versus CB Tconv, we observed higher expression of *LGALS3* and *LGMN*, which enhance *FOXP3* expression (94), as well as *HES1*, which promotes TGF-β signaling (95) (**Figure 4A** and **Supplementary Table S4**). Moreover, in CB Tconv versus APB Tconv, we observed increased *IKZF2*, *TIGIT*, *TNFRSF9* and *SOX4*, the latter of which is induced by TGF-β signaling (96) (**Figure 4A** and **Supplementary Table S4**), supporting the notion that CB Tconv may adopt a more regulatory phenotype than APB Tconv. Interestingly, relative to CB Tregs, APB Tregs expressed higher levels of *GBP1* and *STAT1* (**Figure 4A** and **Supplementary Table S4**), previously shown to be involved in IFN-γ signaling (97) and to serve as a driver of T_H1 differentiation (98), respectively. In addition, compared to APB Tregs, CB Tregs were enriched for markers promoting homing to the gut [*GPR55* (99)], adhesion and migration through the basal lamina [*DST* (100)], and stem-cell and recent thymic emigrant phenotypes [*TCF4* (101) and *THEMIS* (102)] (**Figure 4A** and **Supplementary Table S4**). To summarize these data, heatmaps of a selection of differentially expressed genes between Treg and Tconv show that both CB and APB Tregs highly expressed canonical Treg genes (e.g., *FOXP3*, *IKZF2*, *CTLA4*, and *TIGIT*), while APB Tconv largely lacked expression of these genes (**Figure 4B**). Interestingly, CB Tconv expressed some markers typically attributed to a Treg phenotype, namely *TIGIT*, *IKZF2*, and *FOXP3* (**Figure 4B**), again suggesting a more immunoregulatory phenotype than their APB Tconv counterparts or the selective expansion of Treg following the initial cell isolation. Hence, bulk transcriptomic profiling of CB Tregs supports their lineage stability and retention of a suppressive phenotype post-expansion.

Expanded CB Tregs Exhibit a Highly Activated and Suppressive Phenotype

Next, we expanded CB and APB derived Tregs and Tconv, restimulated with PMA/ionomycin, and examined their phenotype and cytokine production by flow cytometry and Luminex assay, respectively. We found cytokine production and phenotypic profiles to be mostly similar for expanded CB Tregs versus APB Tregs (**Figure 5** and **Figure 6, blue** and **orange**) and for expanded CB Tconv versus APB Tconv (**Figure 5** and **Figure 6, green** and **red**) with the most dramatic differences observed between Tregs and Tconv, regardless of the source (CB or APB). Expectedly, CB and APB Treg produced limited pro-inflammatory and effector cytokines (**Figures 5A–H**) relative to Tconv (103–108), and though we observed no differences in the production of immunosuppressive or effector Treg-associated cytokines (109, 110) between the two subsets (**Figures 5I–K**), we did observe APB Treg to produce increased IL-2 relative to CB Treg (**Figure 5L**). This could be indicative of non-Treg contaminants, consistent with known Treg reliance on exogenous IL-2 (111). Phenotypically, we found CB Tregs to be more activated than APB Treg, as evidenced by an increased frequency of CB Tregs expressing the costimulatory molecule CD226 (**Figure 6A**). Moreover, CB Treg possessed increased proportions of cells expressing CD73 and CD95L (Fas Ligand (FasL) (**Figures 6B,C**), while CD279 (PD-1) was not significantly different between APB and CB Tregs (**Figure 6D**). This suggests CB Treg to have increased capacity for functional suppression via conversion of extracellular ATP to adenosine [CD73 (112)] and activation induced cell death [AICD; FasL (113)], without succumbing to Treg exhaustion [PD-1 (114)]

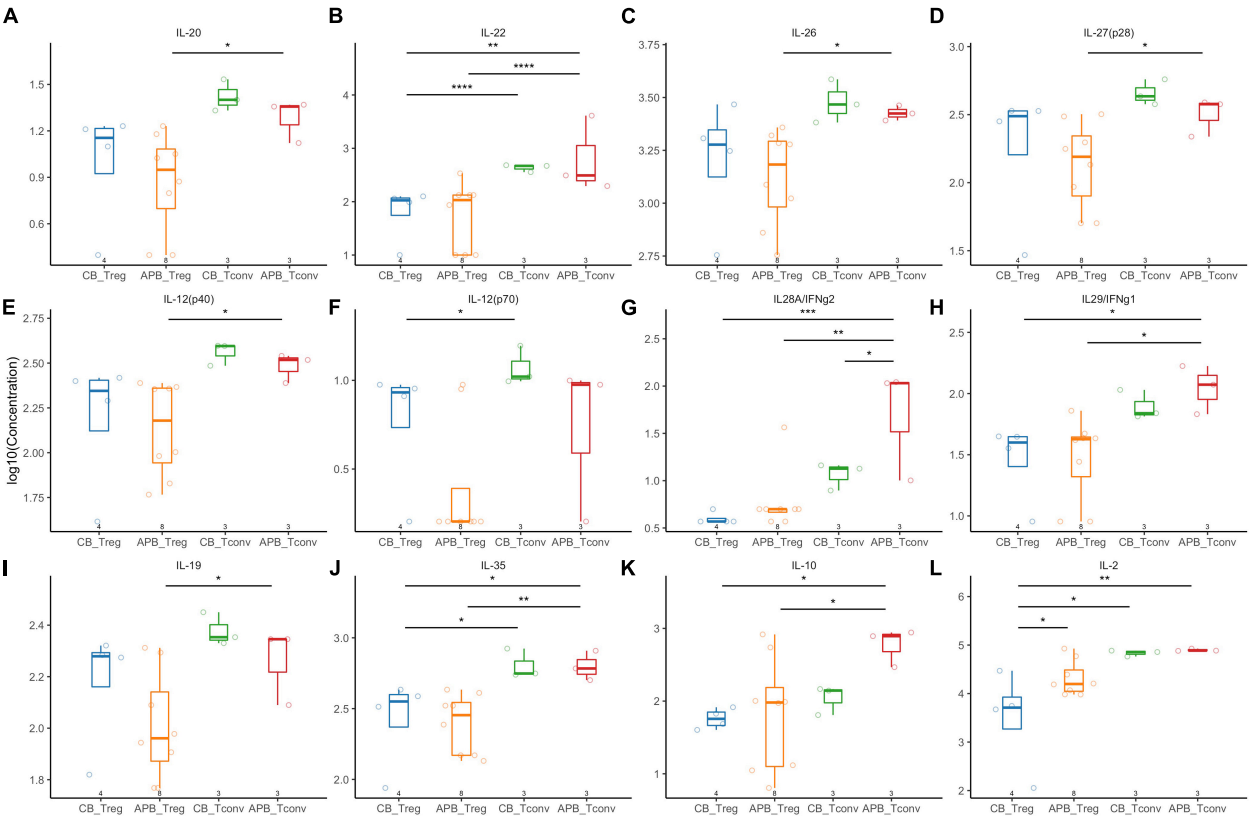

FIGURE 5 | Expanded APB Tregs and CB Tregs have similar cytokine production profiles. Expanded CB Tregs (blue, $n = 4$), APB Tregs (orange, $n = 8$), CB Tconv (green, $n = 3$), and APB Tconv (red, $n = 3$) were activated with PMA/ionomycin and cytokine production detected by Luminex assay. CB Tregs and APB Tregs displayed limited differences in cytokine production profile, as there were no differences in production of **(A)** IL-20, **(B)** IL-22, **(C)** IL-26, **(D)** IL-27, **(E)** IL-12p40, **(F)** IL-12p70, **(G)** IL-28, or **(H)** IL-29. APB and CB Tregs produce similar amounts of immunosuppressive cytokines **(I)** IL-19, **(J)** IL-35, and **(K)** IL-10, while APB Tregs produced significantly more **(L)** IL-2 relative to CB Tregs. When comparing CB Tconv versus APB Tconv, **(A–J,L)** cytokine production was similar except APB Tconv produced significantly more **(K)** IL28A/IFNg2 as compared to CB Tconv. Data were analyzed in R with a mixed effects model using the lmer function in the lme4 package, as noted in Materials and Methods. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(**Supplementary Figure S8**). $CD28^{+}$ cells were more frequent among CB Tregs and APB Tregs as compared to CB Tconv and APB Tconv (**Figure 6E**); this was not surprising given that CD28 signaling is essential for Treg development (115), promotes lineage stability and anti-inflammatory cytokine production (116). CB Tregs and APB Tregs both displayed an increased frequency of cells expressing the activation marker HLA-DR as compared to APB Tconv and CB Tconv (**Figure 6F**). Moreover, compared to APB Tconv, CB Tregs, APB Tregs and CB Tconv displayed increased percentages of cells expressing the suppressive and activation marker, TIGIT (**Figure 6G**), in agreement with our post-expansion bulk sequencing data (**Figure 4**). Compared to APB Tconv, Tregs derived from either APB or CB were enriched for cells expressing CD95, a memory Treg marker (**Figure 6H**) (117). CB Tregs, APB Tregs, and CB Tconv also contained a greater percentage of cells positive for the chemokine receptors CD197 and CD194 as compared to APB Tconv (**Figures 6I,J**), potentially reflective of increased homing potential to secondary lymphoid organs and the skin, respectively (118, 119). Importantly, compared to Tconv, CB and APB Treg possess a reduced percentage of cells expressing CD183 (CXCR3), a T_H1-related chemokine receptor (**Figure 6K**). Moreover, we found CB Treg to possess fewer $FOXP3^{+}HELIOS^{-}$ cells pre- and post-expansion, with an increase in CXCR3 (gMFI and percent positive) noted on this subset within APB (**Supplementary Figures S9, S10**). Finally, we assessed CD49b expression as a marker of T_r1 cells, which have been shown to exhibit similarity to T_H1 cells and to express CXCR3 (120). T_r1 differentiation has been reported to occur in the presence of pro-inflammatory cytokines (121, 122), and while these cells have the potential to be potent suppressors, they lose this capability in the absence of IL-10 while still retaining a cytotoxic program (123), thus making their inclusion in an ACT product a potential risk. The frequencies of $CD49b^{+}$ cells were low and did not differ between CB Tregs versus APB Tregs (**Figure 6L**), indicating a lack of T_r1 differentiation (124). Cumulatively, these data when considered in addition to the transcriptional profiles suggest that CB Tregs retained a highly activated status, suppressive phenotype, and distinct homing capacity when compared APB Tregs.

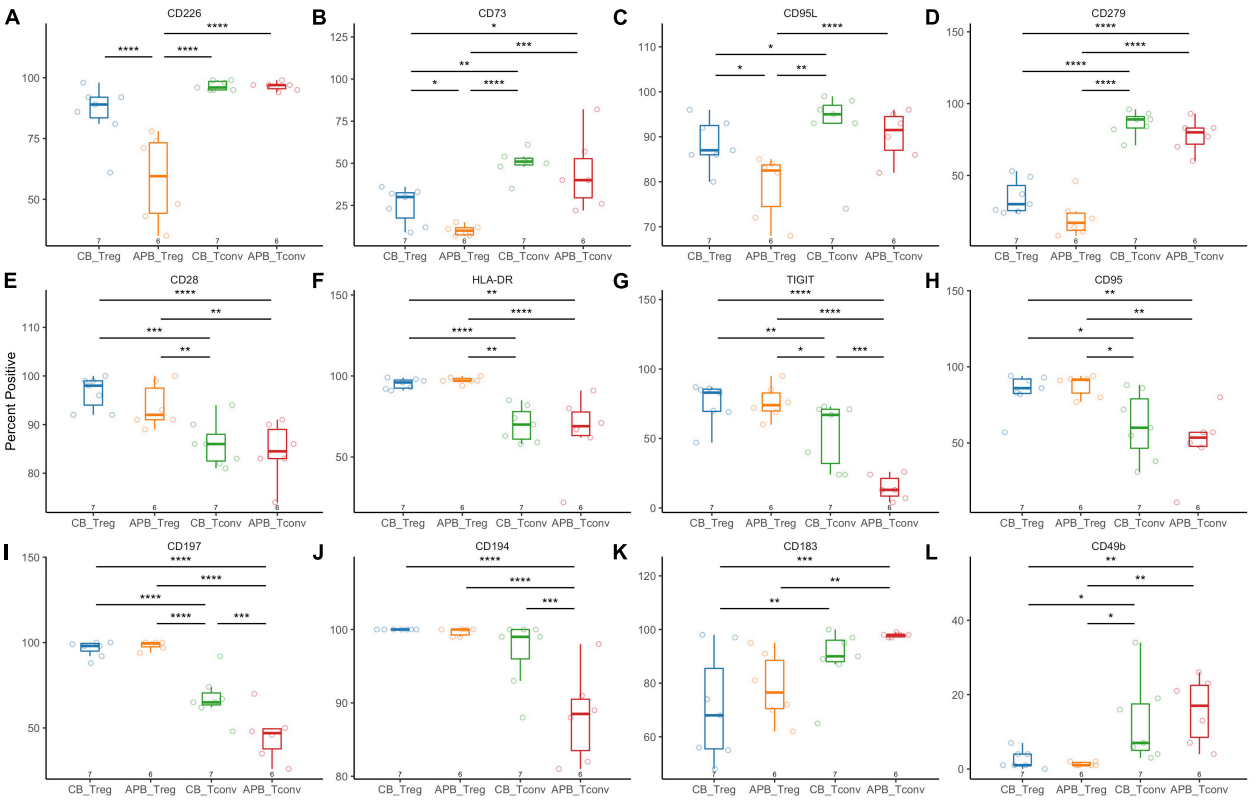

FIGURE 6 | Flow cytometric analysis shows increased activation and suppressive phenotype of CB Tregs compared to APB Tregs. CB Tregs and CB Tconv (n = 7), and APB Tregs and APB Tconv (n = 6) were assessed for differences in phenotype post-expansion. Frequencies of **(A)** CD226, **(B)** CD73, and **(C)** CD95L positive cells were increased in CB Tregs versus APB Tregs. CB Tregs and APB Tregs possessed similar frequencies of **(D)** CD279^{+} (PD-1^{+}) cells, while CD279^{+} cells were more prevalent among CB Tconv and APB Tconv. CB Tregs and APB Tregs also possessed similar proportions of cells positive for **(E)** CD28, **(F)** HLA-DR, **(G)** TIGIT, **(H)** CD95, **(I)** CD197 and **(J)** CD194, with Tregs overall displaying an increased proportion of cells positive for these as compared to Tconv. CB Tconv and APB Tconv displayed an increased proportion of cells expressing **(K)** CD183 (CXCR3) and **(L)** CD49b as compared to Tregs. Data were analyzed in R with a mixed effects model using the lmer function in the lme4 package, as noted in Materials and Methods. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

DISCUSSION

Tregs, when used in the context of ACT, are expected to function as "living drugs" and exert their suppressive functions via numerous mechanisms including expression of negative surface regulators, IL-2 sequestration, and the production of immunoregulatory cytokines as well as other suppressive mediators (125). Importantly, these cells have the potential to traffic to relevant sites of inflammation, and are capable of bystander suppression and infectious tolerance (126). It is precisely this combination of therapeutic properties that has generated great interest in harnessing Tregs for the establishment of long-term tolerance in situations of autoimmunity and/or transplantation. In fact, many of these concepts have been repeatedly demonstrated in animal models of disease but have not, to date, been broadly translated into effective therapies in humans. We would speculate that translation is hampered both by practical considerations (e.g., cost and feasibility of large-scale production) as well as incomplete knowledge of the optimal cellular properties needed to maximize Treg specificity and function in humans.

We previously demonstrated the potential to isolate and expand previously cryopreserved CB-derived Tregs with increased lineage stability relative to APB (32). Given that it is now possible to store CB to create large population biobanks, it is now feasible to consider options for both autologous and HLA-matched allogeneic CB sourcing for ACT applications. Additionally, the utilization of banked CB units avoids the necessity for large blood draws or leukapheresis procedures to obtain sufficient Treg quantities for expansion. This would be highly desirable in pediatric and autoimmune subjects, many of whom may exhibit lymphopenia, increased inflammatory cell populations as potential contaminants, or express multiple genetic susceptibility alleles that may negatively affect Treg function (127–129).

In an effort to better understand how CB compare to APB Tregs, we conducted scRNA-seq of pre- and post-expansion Tregs (**Figure 1**). This analysis demonstrated an enriched cell cluster (herein referred to as C06) in APB defined by a gene expression profile associated with the T_H1 (*CCL5*, *CXCR3*, *BHLHE40*, *NKG7*, *IFNG*, *TBX21*) and T_H17 (*KLRB1*, *TGFB1*, *IL12RB2*, *CCR6*, *RORC*) lineages, as well

as activation and cytotoxicity (*GZMK*, *LYAR*), indicating this population may belong to the recently characterized $T_H1/17$ lineage (66). Interestingly, functional Treg markers (*FOXP3*, *IKZF2*) were downregulated in this cluster amongst APB but not CB Tregs, supporting the notion that CB Tregs are a more homogenous population with increased lineage stability and reduced Teff contaminants. Enrichment of cells expressing pro-inflammatory genes, namely *IFNG*, could have negative implications for Treg plasticity and function. Indeed, IFNγ^+ Tregs have been documented in healthy donors but are enriched in various autoimmune conditions including multiple sclerosis (130) and T1D (31), and have impaired suppressive capacity (131). Moreover, CCL5 and CXCR3 have been shown to be induced following IFNγ signaling (132, 133) and to influence the trafficking of GvHD-promoting proinflammatory T cells (134, 135); hence, we propose that the cellular phenotype of C06 present in APB Treg should be further investigated for its potential to negatively impact the success of Treg ACT.

An important consideration in the development of Treg therapies is antigen specificity, as autoreactive thymocytes with lower to moderate affinity TCRs are thought to preferentially differentiate to the Treg lineage in an autoimmune regulator (AIRE)-dependent manner (136). This bias toward self-reactivity represents a critical paradigm in the suppression of autoreactive Teff in the periphery. However, achieving antigen-specific Tregs in doses sufficient for clinical translation has been hampered by the low frequency of these cells in circulation. We previously demonstrated increased TCR β-chain repertoire diversity in CB Tregs (32). In this study, we expand on those bulk sequences to investigate paired gene expression and TCR profiles within the 6 identified APB and CB Treg clusters (**Figure 2**). We show APB Tregs to demonstrate increased clonal expansion as compared to CB Treg. We were able to define by gene expression the most expanded cluster in APB (C03), which expressed genes indicative of an activated memory phenotype (*S100A4*, *DUSP4*, *S100A10*, *LGALS1*, *LGALS3*). In contrast, the clusters that were moderately expanded in CB represent activated, functional Tregs expressing markers conferring adhesive and migratory potential, namely C01 (*JUNB*, *DUSP2*, and *ITGB1*), C02 (*IDI1*, *FCRL3*, *ID3*), and C06. Importantly, while C06 was shown to function as a contaminant in APB, increased co-expression of the cluster-defining genes (*CCL5*, *GZMK, CXCR3, LYAR*, and NKG7) with *FOXP3* and *IKZF2* in CB compared to APB suggests C06 CB Tregs to retain a regulatory phenotype more so than C06 in APB Tregs (**Figure 2**). We also show an increased number of CB TCRs map to putatively annotated autoreactive sequences, relative to TCRs from APB. Hence, polyclonal CB Tregs might provide a more comprehensive repertoire from which to seed the periphery. Indeed, a recent study has demonstrated the capacity to expand proinsulin (PI)-reactive Tregs from CB with increased yield compared to peripheral blood from subjects with T1D (137). The resultant pool of PI-specific Tregs was found to maintain lineage stability and suppressive function. These results coupled with our data support the notion that CB Tregs represent a population with increased phenotypic homogeneity alongside increased receptor diversity compared to APB Treg, thus serving as an ideal candidate for ACT in autoimmune diseases. Additionally, the potential to generate TCR redirected or chimeric antigen receptor (CAR) Treg has become a topic of great interest in the immunotherapy space (138–140). Our data suggest that the phenotypic stability and homogeneity of CB Tregs relative to APB might ameliorate concerns over lineage stability with TCR or CAR-directed Treg therapies.

We next examined the bulk transcriptomic profiles of CB and APB Tconv and Treg subsets after a 14-day expansion period (**Figure 4**). As expected, APB Tregs displayed increased expression of immunoregulatory markers (*FOXP3*, *IKZF2*, *TIGIT*, *TNFRSF9*) as compared to APB Tconv. In APB Tconv, we observed upregulation of *GZMA*, *GZMB*, *GNLY*, *IL7R*, and *IL18RAP*, which promote pro-inflammatory signaling (141–143) and have been associated with reduced suppressive capacity (144) as well as autoimmune susceptibility (145). In fact, a number of these genes have been implicated in the progression of autoimmune diseases, including T1D (146), hence avoiding cellular contaminants that express them is paramount to the development of an effective therapy with low risk of exacerbating the underlying pathology. While CB Tregs upregulate genes that promote regulatory function relative to CB Tconv (*LGALS3*, *LGMN*, and *HES1*), our data also support a more immunoregulatory phenotype in CB Tconv versus APB Tconv, as evidenced by upregulation of *IKZF2*, *SOX4*, *TIGIT,* and *TNFRSF9*. This observation suggests that, even in naïve CD4$^+$ Tconv subsets, the default developmental program during the perinatal period may preferentially induce a regulatory gene expression profile. Furthermore, we observed increased expression of *GBP1* and *STAT1* by APB Tregs as compared to CB Tregs, likely signifying a more pro-inflammatory phenotype (147) and potentially, reduced suppressive capacity (148). In contrast, CB Tregs were enriched in markers that promote homing to the gut and activation and migratory potential (*GPR55*, *DST*) as well as stem-cell and recent thymic emigrant phenotypes (*TCF4*, *THEMIS*). Interestingly, single nucleotide polymorphisms (SNPs) within the chromosome region containing *THEMIS* have recently been associated with younger age at T1D diagnosis (149), suggesting that alterations in this gene may contribute to aberrant thymocyte selection and thereby, autoimmunity. Collectively, our data imply that expanded CB Tregs may better maintain their ability to traffic to sites of inflammation without the acquisition of an ex-Treg phenotype observed to be enriched in APB Tregs in T1D (28).

In examining Treg surface phenotype, we showed both APB and CB Tregs to express more markers of Treg activation/suppression than Tconv, namely TIGIT, HLA-DR, and CD28. Indeed, TIGIT$^+$ Tregs have been shown to be more activated, to express early activation molecules such as CD69 and checkpoint molecules such as PD-1, and to suppress CD8 T cell and NK cell responses (150). Similarly, HLA-DR$^+$ Tregs have been identified as a highly suppressive population, which is depleted in patients with acute post-transplant rejection (151). PD-1 expression was reduced amongst both APB and CB Tregs as compared to Tconv. While PD-1 expression has been shown to facilitate Treg activation and suppression of Teff responses through interaction with PD-L1 (152), high levels of PD-1 expression have been associated with T cell exhaustion (153).

Indeed, Tregs expressing high levels of PD-1 have been shown to exhibit functional impairments, such as reduced suppressive capacity and increased IFNγ secretion (154). We recapitulated our previous observations in **Figure 4** that show CB Tconv exhibit an immunoregulatory phenotype, with increased TIGIT as compared to APB Tconv. Finally, increased CD226 expression concomitant TIGIT in CB Tregs indicates increased activation, a finding consistent with increased cell yield following expansion cultures (32), while increased CD73 and CD95L expression is indicative of a more broadly suppressive population.

Our data suggest altered transcriptional profiles and suppressive properties of CB Treg relative to APB; however, we acknowledge a number of limitations in our current study. First, our analyses include comparisons across different donors due to longitudinal sample limitations and availability. It is possible that some of the observed differences in gene expression and phenotype are driven by genetic background of donors, and not a function of CB versus APB Tregs. We also acknowledge that some of our observations could relate to the relative imbalance in naïve and memory Treg subsets in CB and APB. Despite these limitations, we expect that the epigenetic profile of CB and APB may differ dramatically, even among the naïve Treg subset. Thus, studies are ongoing to examine the single cell differential chromatin accessibility profiles of CB and APB Tregs through the single cell Assay for Transposase Accessible Chromatin with sequencing (scATAC-seq). These data may also help to uncover the molecular basis for the more regulatory phenotype we observed in CB Tconv.

Our data suggest that modifications of the standard Treg sorting protocol to exclude cells expressing the surface marker CXCR3 present in our APB "contaminant" population as well as targeting/suppressing genes encoding pro-inflammatory markers and transcription factors (*BHLHE40*, *GBP1*) may result in a purer Treg population for use in ACT. Cumulatively, our observations suggest that APB-derived Tregs contain subsets of more differentiated and expanded Tregs as well as contaminants capable of producing cytotoxic molecules and proinflammatory cytokines, which could compromise the success of Treg ACT. In contrast, the CB Treg transcriptomic profile was more homogenous, supporting an undifferentiated regulatory phenotype, and reflects a predisposition for increased cell cycling and proliferation. In sum, clinical CB biobanks may serve as an important source material as the field continues to explore advanced cellular therapies, including the potential for highly specialized and gene and receptor edited cell products to induce durable immune tolerance.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO repository, with the accession numbers GSE137301 and GSE147794.

AUTHOR CONTRIBUTIONS

KM and LP researched and analyzed the data and wrote the manuscript. WV, AE, HS, and ML researched and analyzed the data and reviewed/edited the manuscript. HB analyzed the data and contributed to discussion. AP reviewed/edited the manuscript and contributed to discussion. MB and DP researched the data, contributed to discussion, and reviewed/edited the manuscript. RB analyzed the data and reviewed/edited the manuscript. JL reviewed/edited the manuscript. MH conceived of the study and reviewed/edited the manuscript. TB conceived of the study and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00611/full#supplementary-material

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REVIEW
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Molecular Insights Into Regulatory T-Cell Adaptation to Self, Environment, and Host Tissues: Plasticity or Loss of Function in Autoimmune Disease

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There has been much interest in the ability of regulatory T cells (Treg) to switch function *in vivo*, either as a result of genetic risk of disease or in response to environmental and metabolic cues. The relationship between levels of FOXP3 and functional fitness plays a significant part in this plasticity. There is an emerging role for Treg in tissue repair that may be less dependent on FOXP3, and the molecular mechanisms underpinning this are not fully understood. As a result of detailed, high-resolution functional genomics, the gene regulatory networks and key functional mediators of Treg phenotype downstream of FOXP3 have been mapped, enabling a mechanistic insight into Treg function. This transcription factor-driven programming of T-cell function to generate Treg requires the switching on and off of key genes that form part of the Treg gene regulatory network and raises the possibility that this is reversible. It is plausible that subtle shifts in expression levels of specific genes, including transcription factors and non-coding RNAs, change the regulation of the Treg gene network. The subtle skewing of gene expression initiates changes in function, with the potential to promote chronic disease and/or to license appropriate inflammatory responses. In the case of autoimmunity, there is an underlying genetic risk, and the interplay of genetic and environmental cues is complex and impacts gene regulation networks frequently involving promoters and enhancers, the regulatory elements that control gene expression levels and responsiveness. These promoter–enhancer interactions can operate over long distances and are highly cell type specific. In autoimmunity, the genetic risk can result in changes in these enhancer/promoter interactions, and this mainly impacts genes which are expressed in T cells and hence impacts Treg/conventional T-cell (Tconv) function. Genetic risk may cause the subtle alterations to the responsiveness of gene regulatory networks which are controlled by or control FOXP3 and its target genes, and the application of assays of the 3D organization

of chromatin, enabling the connection of non-coding regulatory regions to the genes they control, is revealing the direct impact of environmental/metabolic/genetic risk on T-cell function and is providing mechanistic insight into susceptibility to inflammatory and autoimmune conditions.

Keywords: Treg FOXP3, gene regulation, genetic risk of disease, T-cell fate, T-cell plasticity

INTRODUCTION

Establishing and Maintaining Immune Homeostasis

To maintain health, the immune system continuously and dynamically balances robust reactivity against pathogens with tolerance or unresponsiveness to self-antigens, commensal bacteria, food, and external harmless antigens (1). This is in part mediated by the effector arm of the adaptive immune system, and two of the major T-cell mediators of this are CD8 and CD4 cells. These are selected based on their ability to respond to antigens presented on either MHC class 1 or 2, respectively. The CD4+ T-cell compartment comprises a growing number of specific effector subsets, each of which is programmed to respond to defined antigen families and home to specific locations. Antigen specificity is determined by the affinity and avidity of T-cell receptors (TCRs). This specificity is generated during CD4/CD8 commitment, differentiation, and selection in the thymus, in a process that includes deletion of (strongly) self-reactive TCR-bearing T cells as a mechanism to prevent autoreactive TCR-bearing clones being released into the periphery. As no biological process is 100% efficient, there is the potential for self-reactive T cells to escape selection and hence be released into the periphery. To manage this, regulatory T cells (Treg) are also generated with the same TCR specificities. Treg are selected in the thymus but also generated from naïve T cells in the periphery. Both thymic (nTreg) and induced (pTreg) Treg are similar in function, but they have different roles and targets cells (2, 3). The key difference between these subsets is that pTreg provide immune surveillance of specific organs and biological processes in the periphery for which there is no inherited specificity, such as tolerization of the conceptus in pregnancy or the bacteria and food antigens in the gut by pTreg (3).

Roles and Function of Treg

In a general sense, Treg act as "policemen" of the immune system to limit rogue immune activity, and this role in immune homeostasis is critical. In addition to regulating antigen-specific immune responses, Treg are capable of regulating cell function in an antigen-independent manner (4) and are now implicated in tissue homeostasis and repair (5–8). Treg actively control the proliferation and activation of cells of both the adaptive and innate immune systems and achieve this using multiple mechanisms, which are tailored to the environment in which they are required to function (1). The suppressor mechanism is likely to differ according to the physiological and inflammatory state encountered (1, 9). While autoimmunity and chronic inflammation are accepted to arise as a general failure of tolerance, given that the effector and Treg arms of the system need to be in balance, this can occur because of numerical reduction in Treg, functional reduction in Treg potency without reduced numbers, expansion of effector T cells, or T effector resistance to suppression. In order to examine this at high resolution in clinical cohorts, Treg-specific biomarkers are essential. The first biomarker for Treg was CD25, the IL2 receptor alpha chain, which gained widespread recognition as stable expression of the IL2 receptor (CD25) tracks with the suppressor function in CD4+ T cells (10). Many groups have further characterized CD25 expression and conclude that CD25 expression is strongly upregulated on Treg, but transient activation of CD4+ T cells can induce CD25 without inducing regulatory function, so it is not an exclusive Treg functional marker. The definitive biomarker of suppressor function is FOXP3, but because it requires an intracellular stain with a protocol to fix and permeabilize cells, viable cells cannot easily be recovered, making it less tractable as a live cell biomarker. In search of surface-expressed surrogates of the suppressor function or FOXP3 expression, two groups observed that reduced expression of the IL7 receptor (CD127) is a hallmark of the human Treg phenotype (11, 12). However, as activated murine Treg express CD127 strongly (13), CD127 is not selective for mouse Treg. Deeper interrogation of the function of Treg subsets is suggesting that differential expression of other cytokine/chemokine receptors on Treg may be useful for tracking Treg *ex vivo*. A growing number of other cell surface markers are found on specific Treg subsets, e.g., TIGIT (14–16), FcRL3 (17), GARP/LRRC32 (18–21), CD73 and CD39 (22, 23), and, more recently, PI16 (24). The mechanism for TIGIT in establishing the suppressor function both directly and indirectly includes induction of tolerogenic dendritic cells (14, 15), and coexpression with FcRL3 marks human memory Treg that express Helios and are highly suppressive (17). Many of these genes are regulated by FOXP3.

Because many of these cell surface molecules are also found on effector cell populations, they are not powerful biomarkers in isolation, and more complexity in the Treg phenotype exists than two parameter biomarker combinations suggest. The use of new single-cell transcriptomic approaches (25) and high-resolution cytometry (26, 27) is enabling better resolution of the coexpression of marker genes in these low-abundance Treg subsets.

Molecular Mechanisms Shaping Treg Stability and Phenotype

FOXP3 is the key transcription factor for the formation and function of Treg in mice and humans (28–30). Genomics including RNAseq and chromatin immunoprecipitation (ChIP)

has helped identify the molecular basis for the action of FOXP3 in shaping regulatory T cells and in establishing and maintaining lifelong tolerance. While loss of Treg function is observed in a wide variety of autoimmune and chronic inflammation states, there remains the possibility that the loss of function is a consequence of, and not the cause of, autoimmunity and chronic inflammation. It is clear that Treg lineage formation is dependent on FOXP3, which establishes and maintains suppressor function. With additional analysis of FOXP3 cell origins in mice, the existence of two FOXP3+ve Treg populations of different ontologies has revealed the mechanism for tolerance induction in the periphery. Natural or thymic Treg emerge from the thymus stably expressing FOXP3 and fully mature. In contrast, peripheral Treg arise from FOXP3-negative naïve T cells which do not express FOXP3 until stimulated in the presence of cytokines and transcriptional activators, which turn on the FOXP3 gene. The molecular steps required to set up and stabilize the expression of FOXP3 in the thymus, including a key role played by SATB1 (31–33) and Helios (34–39), may be distinct from those inducing FOXP3 in the periphery. Recently, the role of Helios in Treg ontology was further elucidated as deficiency in Helios results in preferential differentiation into pTreg (35). Once established, many of the gene regulatory networks (GRNs) controlled by FOXP3 are similar in pTreg and tTreg, and subtle functional gene networks are set up to shape lineage restriction or maturation state. Hence, using similar molecular mechanisms, the Treg compartment has the ability to acquire tolerance to antigens from inherited repertoires, such as self-tissues and organs (tTreg), and to *de novo* antigen exposure, such as pregnancy alloantigens, commensal bacteria, food, and chemicals (pTreg).

Control of Expression of FOXP3

During formation of Treg in the thymus, the FOXP3 locus is set up for active transcription by chromatin remodeling (31, 32), and the protein SATB1 is implicated in initiating this. Other transcription factors, including FOXP1 (40), are also required to set the stable expression of FOXP3. The expression of FOXP3 is impacted at the transcription and posttranscriptional levels, and this is sensitive to reversible processes including methylation (41–48) and acetylation (49–51). Additional regulation of FOXP3 gene expression is influenced by non-coding RNA-mediated mechanisms (33, 52–59). The regulation of transcription of FOXP3 by distinct modules including the Treg-specific demethylated region (TSDR) (41, 42, 60) has revealed marks for FOXP3 expression control and can discriminate between thymic Treg FOXP3 expression and activation-dependent expression of FOXP3 in naïve T cells in the periphery (41, 47). The methylation or demethylation of the TSDR is controlled by DMT3 or TET, respectively, and this process is regulated tightly in both thymic induction of FOXP3 and induction of FOXP3 in the periphery (61–63). Detailed functional mapping of the FOXP3 locus regulatory elements has defined specific regions near the TSDR identified as conserved non-coding sequences (CNS) 1, 2, and 3 (64). CNS1 restricts expression of FOXP3 to iTreg. CNS2 includes the TSDR and drives maintenance of FOXP3 in all Treg, and CNS3 is responsible for FOXP3 expression in thymic Treg (64). Specific transcription factors bind at each region, including AP1 and NFAT at CNS1 (60), Runx1 and CBFβ at CNS2 (65), and cRel at CNS3 (64). Activation-induced expression of FOXP3 in naïve human CD4+ T cells (66–68) results from partial but not complete demethylation of the FOXP3 locus, generating iTreg (41). In the presence of TGFβ and all-trans retinoic acid (ATRA) (64), the expression of FOXP3 is stabilized to some degree. Hence, the relative methylation state of the FOXP3 regulatory elements (CNS1, CNS2, and CNS3) is a potential axis for Treg plasticity.

Molecular Identification of the FOXP3 Regulome

Understanding the mechanisms of transcriptional control of the Treg suppressor genotype by FOXP3 has been increased by ChIP experiments, which crosslink transcription factors bound to genomic DNA. Genome-wide mapping of FOXP3-binding sites provides insight into the regulation of the genes that shape the Treg phenotype. In human Treg, of the 2,000–3,000 regions bound by FOXP3 identified by our and other FOXP3 ChIP experiments (57, 58, 69, 70), only a subset of the FOXP3-bound regions maps to genes that are directly differentially expressed or repressed in human Treg at any given time, including SATB1 (33). FOXP3 ChIP studies have identified a significant number of loci in mouse and human Treg that are directly bound by FOXP3 and can be annotated to differentially expressed Treg genes (**Figure 1**). However, many loci either were too far from a transcription start site to annotate to a target gene easily or do not appear to be associated with differentially expressed genes in Treg. This can be explained because there are a number of differentially expressed genes in Treg that are indirect targets of FOXP3 or are controlled by FOXP3-induced miRNAs. For example, in our human FOXP3 ChIP dataset, only 750 of almost 3,000 FOXP3-bound regions were annotated to a differentially expressed gene in human Treg (57). This revealed a network of core genes that are tightly regulated by FOXP3. However, there is a limitation of linear models of nearest-neighbor annotation, as it does not capture interactions that occur as a result of DNA looping. Nonetheless, specific genes interact with FOXP3 to form the FOXP3 GRN, and this GRN shapes the function of Treg.

Multiplexed Transcriptional Control of T-Cell Function

Each helper lineage in the CD4 pool has a defining transcription factor, the expression of which shapes lineage-restricted function. As previously stated, FOXP3 controls the GRN essential for suppressor function, but this also acts in the context of the lineage-defining transcription factors. There can hence be a second or third partner transcription factor working in cooperation with the lineage-defining master regulator. These transcription factors can be induced by specific external stimuli, including cytokines and growth factors, metabolites, and cell contact-mediated signals. As a result, a transcriptional program is established which enables the cell to express pathogen-specific effector molecules and follow pathogen-specific homing cues. This raises the possibility that terminal differentiation and

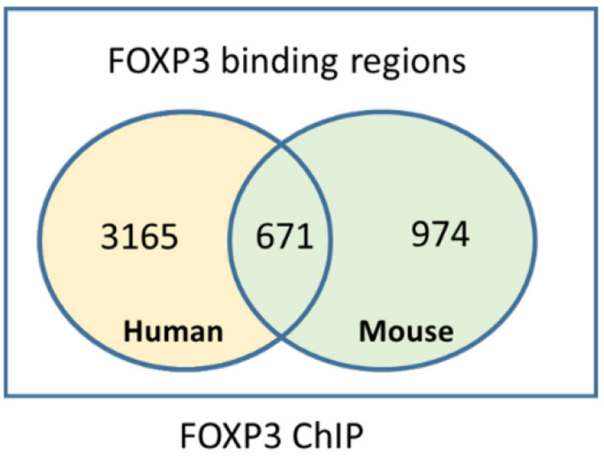


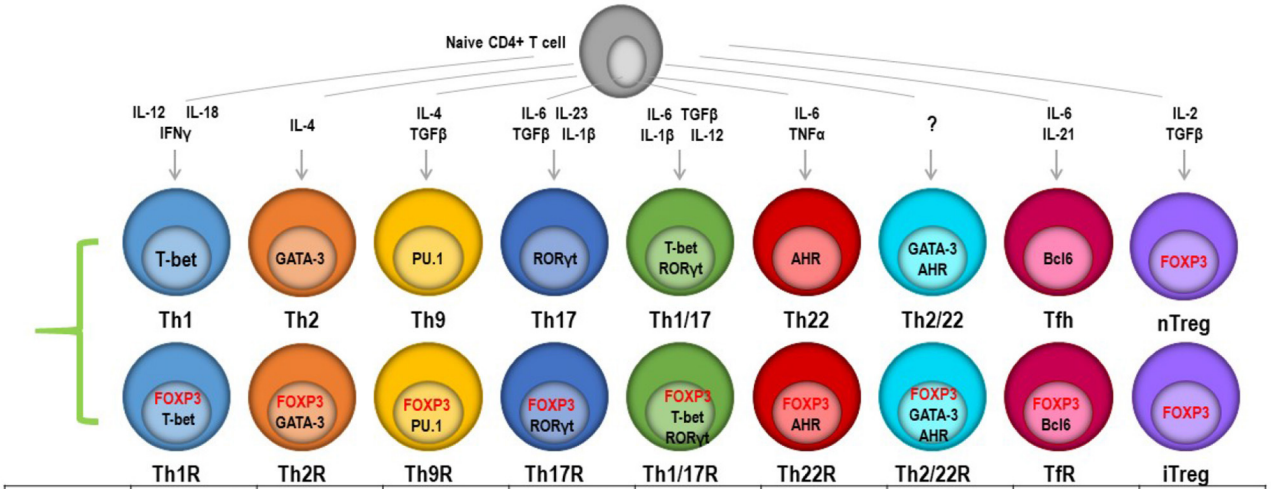
FIGURE 1 | Intersection of mouse and human FOXP3 target genes identified by chromatin immunoprecipitation.

function may not be predetermined or fixed in a given lineage but is reprogrammable. A requirement for plasticity in T-cell responses may be 2-fold; it may be part of a mechanism to quell an active immune response once the pathogen has been cleared. Alternately, functional plasticity may enable multiple tailored responses from a common progenitor, giving the option of a response tuned to the challenge type and site. An emerging theme is that the Treg compartment is paired with the conventional T-cell (Tconv) effector compartment so a matching Treg can regulate any immune response mediated by any T-cell subset (24, 71–73). This is supported by the detection of lineage-specific transcription factors [e.g., Tbet (74) and IRF4 (75)] coexpressed with FOXP3 in Treg subsets, and this has been validated in various mouse models (**Figure 2**). The application of single-cell RNAseq, CITEseq, and other high-resolution transcriptomics on highly purified human Treg subsets is enabling the identification of more signature molecules for each functional subset. Single-cell transcriptomics has recently been applied to Treg from lymphoid and non-lymphoid tissues to compare differences and similarities in rare tissue-homing populations, and this reveals common non-lymphoid tissue-specific signatures in Treg but also that there are significant differences between these subsets in mice and humans (25). The use of functional assays to interrogate highly purified cell populations then allows the question of altered committed lineage proportions vs. plasticity to be better understood.

Non-coding RNAs: Rheostats of Treg Gene Expression

MicroRNAs (miRNAs) are 21–22-nt, non-coding RNAs often found within the introns of genes. They can posttranscriptionally regulate gene expression by either cleavage of the mRNA transcript or inhibition of translation. The impact of a small number of regulated miRNAs is significant because a single miRNA can target and regulate multiple genes. Likewise, multiple miRNAs can target an individual mRNA transcript, giving rise to complex regulatory networks and fine-tuning of gene dose. Rather like mRNAs, miRNAs are also both direct and indirect FOXP3 targets in Treg, and a subset of these miRNAs is also differentially expressed, suggesting a key role for miRNAs in Treg function (76). Selective inactivation of miRNA processing by deletion of the Drosher gene in Treg induced a lethal inflammatory disease in mice, due to a significant reduction in Treg numbers (52), suggesting that miRNAs are required for Treg formation. Furthermore, using Dicer-knockout mice, Liston et al. showed that Treg-suppressive function is absolutely dependent on miRNA biogenesis. These mice showed significantly reduced suppressive activity (54). Further validation of the importance of miRNAs in shaping Treg function was provided by Zhou et al., who demonstrated that depletion of mature miRNAs led to uncontrolled autoimmunity and skewing of iTreg to a Th1/Th2-like effector phenotype. Interestingly, no effect was seen in tTreg (59). This established the possibility that miRNAs may confer a rheostat-like function in T-cell lineage differentiation and plasticity. The potential for the cooperation of miRNAs and FOXP3 enabling tight control of Treg phenotype and function is mechanistically plausible. We and several other groups have demonstrated that miRNAs, such as miR-155 and FOXP3, cooperate to coordinately repress other key genes in Treg and other cell types (77), including SATB1 (34) (**Figure 3A**), and have identified a number of other candidate miRNAs involved in reinforcing the Treg genotype. We observe that a common miRNA/FOXP3-mediated molecular switch is able to regulate several key genes, and this forms a negative feedforward component shaping part of the FOXP3 GRN.

There is also evidence for positive feedforward regulatory mechanisms in Treg, whereby FOXP3 induces genes while also repressing miRNAs that can target that gene (**Figure 3B**). A positively regulated miRNA signature for human nTreg feedforward loops includes miR-21, miR-155, miR-125a, miR-146a, miR-181c, and miR-374. There are miRNAs that can target FOXP3 itself, including miR-15a/16 (78), miR-24, and miR-210 (79); however, less is known about the miRNAs which form part of a negative regulatory loop. An miR-31 target sequence in the 3′-untranslated region (UTR) of FOXP3 suggests that miR-31 may be able to negatively regulate FOXP3, and this was validated by overexpression in cord blood nTreg, resulting in a significant reduction in FOXP3. In contrast, antagonism of miR-31 leads to increased expression of FOXP3, suggesting that miR-31 directly regulates FOXP3 (56). miRNA-31 was not identified in the mouse miRNA Treg signature (52), but Zhang et al. identified a potential FOXP3-binding site within the promoter region of the gene encoding murine miR-31 (80), suggesting that FOXP3 may directly target miR-31. Semiquantitative RT-PCR of miR-31 was ∼90-fold higher in Tconv than in Treg, and mouse FOXP3 ChIP demonstrated occupancy at the miR-31 promoter (81). Taken together, these experiments suggest that FOXP3 can bind to and downregulate expression of miR-31 in Treg, and by performing an alignment with the human miR-31 host gene, there is also a comparable FOXP3 consensus binding site in human miR-31. Given this potential regulatory rheostat relationship between FOXP3 and miR-31, it is interesting that miR-31 is dysregulated



	Th1R	Th2R	Th9R	Th17R	Th1/17R	Th22R	Th2/22R	TfR	iTreg
Cytokines	IFNγ, GM-CSF, IL-2, LTβ, CCL2	IL-4, IL-5, IL-13	IL-9, IL-10, IL-21	IL-17, GM-CSF, IL-21, IL-22	IFN-γ, IL-17, IL22	IL-22	Unknown	IL-4, IL-21, IFNγ	IL-10, TGFβ
Cell surface phenotype	CD4+ CD25- CD127+ CXCR3+ CCR6-	CD4+ CD25 CD127+ CXCR3- CCR6- CCR4+ CCR10-	CD4+ CD25- CD127+ ?	CD4+ CD25- CD127+ CXCR3- CCR6+ CCR4+ CCR10-	CD4+ CD25- CD127+ CXCR3+ CCR6+	CD4+ CD25- CD127+ CXCR3- CCR6+ CCR4+ CCR10+	CD4+ CD25- CD127+ CXCR3- CCR6- CCR4+ CCR10+	CD4+ CD25- CD127+ CXCR5+	CD4+ CD25+ CD127-
Function	Immunity to intracellular parasites. Implicated in pathogenic inflammation.	Immunity to helminths. Involved in allergic responses.	Immunity to helminths. Involved in allergic responses.	Immunity to extracellular pathogens and fungi. Implicated in pathogenic inflammation.	May be implicated in pathogenic inflammation.	Direct immune responses in the skin.	Unknown	Required for class-switching and affinity maturation of B cells.	Immune-suppressive. Protect against pathogenic autoimmunity.

FIGURE 2 | Transcription factor-controlled function in both effector and Treg lineages gives rise to paired Treg to match effectors. These can be resolved by combinatorial chemokine receptor profiling and are predicted to be able to follow the same cues into specific tissues to manage a pathogen and the restoration of homeostasis once the pathogen is eliminated.

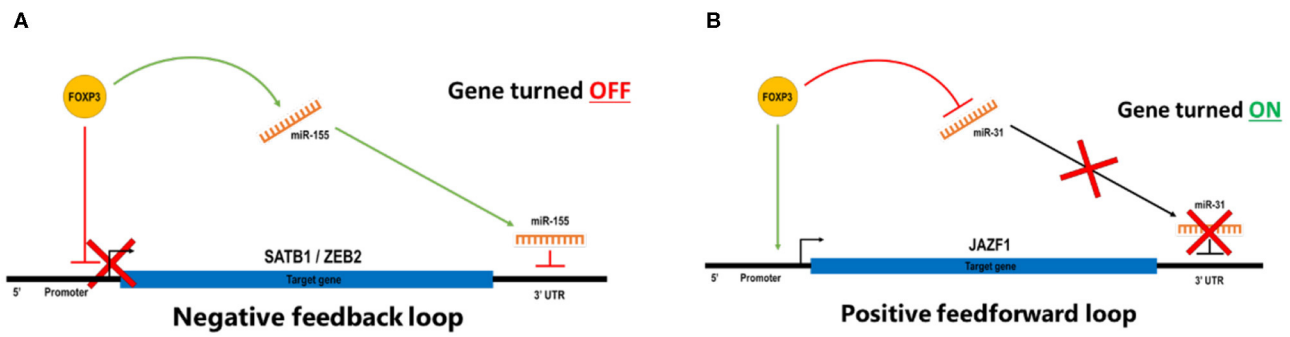


FIGURE 3 | (A) In a negative feedback loop, FOXP3 binds directory to the target genes to repress transcription and also induces miRNA that targets the 3′ UTR of the same genes to degrade transcripts or blockade of translations. **(B)** In a positive feedforward loop, FOXP3 binds directory to the target genes to induce transcription and also represses miRNA that targets the 3′ UTR of the same genes to prevent degradation of transcripts or blockade of translations. In a stable Treg, FOXP3 represses miR-31 by direct binding to regulatory elements associated with the gene, and FOXP3 also targets the promoter of a suppressor function reinforcing the gene to turn it on. In effector T cells, miR-31 expression prevents the expression of FOXP3 by targeting FOXP3 mRNA for degradation.

in several autoimmune diseases such as inflammatory bowel disease (IBD) or Crohn's disease (82, 83) and Kawasaki disease (84). Hence, identifying the molecular mechanisms by which FOXP3 and miR-31 regulate each other and identifying the other downstream target genes in this regulatory network could assist in the development of novel treatments for autoimmune diseases.

Lineage Fidelity and miRNAs

Sentinel transcription factors in each lineage and the miRNAs controlled by them shape CD4+ T-cell phenotypes. An example of the involvement of miRNAs in this is the targeting of mTOR by miR-99a and miR-150 (85), which skews metabolic processes and influences levels of FOXP3 and RORgamma (86). This may enable switching between functional phenotypes by driving one

transcription factor to decline and another to dominate, which is a potential molecular mechanism for plasticity. In addition, it is interesting to speculate that the transient expression of FOXP3 in activated Tconv then induces miRNAs, which together transiently repress effector function gene networks, and this enables the effector cells to return to a resting state.

A Role for Long Non-coding RNAs in Shaping Treg Function

In addition to short non-coding RNAs, high-resolution functional annotation of the human and mouse genomes has revealed the prevalence and importance of long non-coding RNAs (lncRNAs), which also act as subtle regulators of gene expression. lncRNAs (defined as non-coding transcripts >200 bp) are not translated but can regulate gene expression as a result of interaction with mRNA and chromatin. This is achieved by either stabilizing DNA looping or by integrating into the RNA-binding protein complex to regulate transcription (87). The lncRNAs have been implicated in differentiation of T-cell subsets (88) and in immune function (89, 90). Alterations in lncRNAs function have been identified in autoimmune and chronic inflammation samples (91). In keeping with miRNA feedforward and feedback loops, the importance of lncRNA in Treg has been elegantly demonstrated for an lncRNA (FlicR) in mouse and human Treg. FlicR stabilizes the expression of FOXP3 (92) via interactions with conserved non-coding elements that control FOXP3 expression, and loss of FlicR results in reduced expression of FOXP3. Although it is not clear what regulates transcription of FlicR itself, controlling FlicR could establish the transcriptional reprogramming of Treg. If its expression was susceptible to external cues such as IL2 signaling, this would support a model that plasticity can be induced by relatively small changes in signaling that result in altered transcriptional networks. It is also interesting that a role for lncRNA in stabilizing DNA looping has been proposed. Consistent with this, lncRNAs are often encoded within enhancer regions, which shape the expression of multiple genes. Since a significant proportion of autoimmune genetic risk is also found in enhancers and the enhancers loop to form the regulatory hubs for key immune function genes, this suggests that there could be a complex network effect on multiple targets from genetic risk at a single lnc/enhancer module (93).

Translational Regulation and Treg Phenotype

In addition to transcription and posttranscriptional regulation of the Treg phenotype, there is a layer of control of the Treg or T effector phenotype at the translational level. This is influenced by activation and TCR crosslinking and is mediated by ribosome occupancy levels on mRNA and the expression of translational machinery, which can differentially impact protein levels in the cell. One member of the transcriptional initiation complex is elongation initiation factor 4E (eIF4E), and its expression inversely correlates with FOXP3 expression (94). A cluster of effector cytokine genes is positively regulated by eIF4E and is expressed by activated Tconv but is repressed in Treg. It is interesting to note that IL2 signaling can induce eIF4E, but in Treg, the expression of FOXP3 can repress this. Given that Treg are dependent on exogenous IL2 for survival and proliferation signals, but Teff can express IL2, there are likely additional layers of regulation that prevent IL2-induced gain of effector function in Treg. This may include the regulation of some of the downstream signaling by mTORC. Hence, a disease-linked alteration of eIF4E levels in Treg is likely to reduce FOXP3 expression and unleash effector cytokine expression, driving the switching of phenotypes (plasticity or immune defect) (95).

Treg Gene Expression Is Set by Enhancer–Promoter Interactions

Genes comprise as little as 1.5% of the human genome, encoding ∼21,000 proteins, leaving 98.5% of the genome that is non-coding, and this is responsible for orchestrating the cell-specific expression of genes required for formation and function of every cell in the body. It is now clear that interactions between coding and non-coding elements are essential for normal gene regulation and maintenance of stable phenotypes. To achieve this in the contact of the relatively compact nucleus, chromatin structure has a major influence on gene expression by controlling transcription factor access to binding sites in enhancers and promoters (96). This is shaped by patterns of chromatin modification at the base pair level, such as DNA methylation, or the macromolecular level, such as histone modification and nucleosome remodeling, and these correlate with transcription factor binding, enhancer activity, and initiation or repression of transcription (97–99). There are additional, highly active enhancer clusters named super-enhancers (100, 101), and they appear to regulate key genes involved in T-cell function. It is now necessary to consider how enhancers interact with their target genes, particularly as they can be significant distances apart on linear DNA. DNA looping promotes gene network formation, and a single enhancer can interact with more than one promoter, and a single promoter may be contacted by more than one enhancer. As this 3D chromatin organization is being unraveled, it appears that many of these interactions occur in a tissue-specific manner and are the major determinants of cell-type-specific responses (102–105).

Transcriptional Control by DNA Looping

DNA looping brings specific genes and regulatory elements together into transcriptionally active hubs (106), and these hubs may be different in Tconv or Treg. However, since DNA looping cannot currently easily be predicted using bioinformatics approaches, proximity-based annotation of FOXP3 targets based on linear DNA organization under-ascribes FOXP3-binding sites in chromatin to transcriptional targets. This has provided a partial explanation for the apparently low intersection of transcription factor ChIP peaks (including FOXP3) with differential expression of target genes in the same cells, as those interactions have traditionally been annotated using a linear nearest-neighbor approach.

New techniques have been developed to solve the problem that bioinformatics alone is not readily able to predict long-range DNA looping. These techniques are collectively known as

chromosome conformation capture (3C) assays, and these are essential to study the role of DNA looping in transcriptional regulation. 3C is able to efficiently crosslink looped DNA for barcoding, and by using sequencing and mapping of non-contiguous sequences, it is possible to directly identify genomic loci that are in interaction partnerships over short and long distances (107). Variants of 3C such as 4Cseq, ChIA-PET (108), and 5C (103) can identify individual promoter interactions (3C), the network of interactions with one bait locus (4C), the interactions between a transcription factor bond to DNA ant its contacts (ChIA-PET), and single-cell conformation capture (5C). These methods have been extended to examine interactions in an unbiased genome-wide manner, HiC (102). HiC enables mapping of whole-genome chromatin interactions, although there is currently little confidence in the statistical power for predicting interchromosomal interactions. As HiC aims to annotate any/all contact between any two loci on a genome-wide level, it requires deep sequencing to generate comprehensive coverage and to generate interaction maps at the resolution required to map both near and far contacts with accuracy. Given that a significant number of interactions involve a promoter, as that is essential for regulating gene expression, modifications of HiC to reduce sequencing depth and to focus the sequencing to regions of functional interest have been derived. This is achieved by adding oligonucleotide capture technology to enrich regions of interest (such as promoters) in the HiC library prior to high-throughput sequencing (109–111). An advantage is that the HiC library can be re-probed with different oligo libraries, e.g., enhancers, promoters, or ChIP sites, and this enables validation of promoter capture by reverse capture from the same cell source (102). ChIP combined with HiC enables generation of specific protein-centric interactome maps (112), known as HiChIP (113). When H3K27ac HiChIP was performed in naïve T cells, Th17, and Treg, it shed new light on the lineage-specific interactome by annotating lineage-specific accessible chromatin interacting with regulatory elements (114). Importantly, 3C-based assays have been used to successfully identify targets of disease-associated variation in many cell types (103, 108, 110, 115) including human CD4 and CD8 populations (116). By adding DNA looping, enhancer annotation, and FOXP3 binding data to genetic risk data, it is possible to filter genetic risk to Treg-specific functional regions using bioinformatics. However, functional validation of these regions still needs to be performed on human Treg.

Chromatin Accessibility Controls Gene Regulation in a Cell- and Activation-Specific Manner

Annotation of active, open, or closed chromatin has facilitated mapping of cell-type-specific gene regulation [e.g., epigenomics roadmap (97, 117) and FANTOM (98, 118–120)]. These consortia have provided additional evidence to map autoimmune disease in the context of the activation state, connectivity, and accessibility of the genome. ATACseq (Assay for Transposase Accessible Chromatin with high-throughput sequencing) probes chromatin accessibility, TF occupancy, and nucleosome positioning with low starting material (121, 122). As compared to other genome-wide chromatin probing methods, ATACseq is relatively simple and rapid. It is also highly sensitive. The requirement of low-input material makes ATACseq amenable to use on rare population and small clinical samples such as biobanked material (123). At a high-sequencing depth ATACseq can also be used to identify TF binding sites at single-base-pair resolution. TF-occupied sites prevent Tn5 cleavage and adaptor insertion, thus leading to protected regions (footprints) in the sequencing reads (121). This technology is enabling the base pair level mapping of the potential impact of genetic risk on gene regulation, as a SNP that alters a TF binding site will alter the profile of the ATACseq signal at that region, compared with the non-variant base at the same locus. In addition, chromatin accessibility profiling in human Treg enables identification of cell-specific accessible regions that contain features such as FOXP3-binding sites and genetic risk, and these can be distinguished from regions nearby that may also contain genetic risk but are not active in the cell type of interest.

Environmental Signals and Transcriptional Programming of T Cells

Sensing External Stimuli at Sites of Inflammation

The process of recruitment of effector cells to pathogens in the host tissues is driven by local tissue cues drawing the cells to the site as well as pathogen recognition signals, and this is in part mediated by the activation of the pro-inflammatory milieu at these sites. This suggests that local signals may contribute to the shaping of the phenotype of the cells once they home to the challenge site (73, 124). It is also common that the tissue site has altered metabolic status, such as hypoxia and altered redox states. It is now evident that T cells are also highly responsive to these metabolic cues, and these are sensed by common surface receptors and biochemical pathways. The potential for altered regulation of the immune response at these sites exists because the Treg have to home to the same locations to regulate the effector response after pathogen has been cleared, and they are also exposed to the same metabolic environment. For robust regulation by Treg in this context, the FOXP3 GRN has to resist environmental fluctuations (125). However, it is also possible that tissue-specific cues can transiently reduce Treg function and limit their suppressive potency, in order to enable pathogen clearance. Taking into account that strong T-cell activation can also induce transient FOXP3 in effector cells, this may be the mechanism by which the effector cells themselves return to a resting state, but this has yet to be proven.

Transcriptomics has revealed micronutrient transporters and receptors on human T cells, rendering them responsive to a wide range of metabolic molecules and signals. These include sugars, amino acids, environmental toxins (126), energy molecules, vitamin metabolites, and food metabolites, many of which are processed by the microbiome. Functional validation and characterization at the molecular levels suggest that these pathways are particularly relevant for induced Treg generation in the gut. These mediators play a key role in differentiation of naïve T cells, but it remains to be definitively proven that they can drive fate change in committed T-cell subsets. Furthermore, T

cells can sense oxygen tension and oxidation state, and these also exert a phenotype-altering potential as they in turn regulate gene networks influencing biochemical responses including glycolysis. As described below, the balance of oxidative phosphorylation vs. glycolysis is linked to transcription of FOXP3 and regulatory phenotypes (127, 128). This in part enables Treg to function in environments that are under oxidative stress (129) and also suggests that metabolic status could skew immune function.

A number of specific metabolites which can alter transcriptional programming and T-cell differentiation have also been identified. For example, FOXP3 can be induced directly in response to short-chain fatty acids processed from complex carbohydrates, e.g., starch, in the colon by specific microbiome constituents. Hence, butyrate processed by commensals in the colon is able to promote a tolerogenic bias (130, 131). Other metabolites that have been well-characterized include the vitamin A metabolite retinoic acid (ATRA) either alone or in combination with other factors. ATRA can induce either Treg or Th17 phenotypes by upregulating FOXP3 expression or RORgamma expression, respectively (132). This tolerance/inflammation axis can also be influenced by sensing toxins and pollutants via the aryl hydrocarbon receptor (AHR) (133, 134). Mechanistic insight comes from functional mapping of the genes and pathways of the sensors of metabolic stimuli, including mTor and HIF (135, 136). The impact of high salt on Treg function and plasticity has been postulated, and a key mediator of responsiveness to high levels of sodium is the serum/glucocorticoid-regulated kinase (SGK-1) (137). The molecular mechanisms of SGK-1-mediated regulation of Treg suppressor function have been validated in knockout models, and this demonstrated that SGK-1 is induced by IL23/IL23R signaling, and elevated levels of SGK-1 result in reduced suppressor function, which is linked to reduced FOXP3 expression, and that is caused by reduced binding of FOXO1 to the CNS1 element in the FOXP3 promoter, described above. The induction of SGK-1 therefore induces a transcriptional bias to Th17 cells. Knockout of SGK-1 results in enhanced Treg function and reduced pathology in EAE models, confirming a functional link to immune tolerance balance (138). All of these pathways and inducer molecules are implicated in altered Treg function or numbers in disease and are potential targets for interventions to restore balance.

Energy Pathways and T-Cell Function

Long-lived quiescent T cells primarily utilize oxidative phosphorylation pathways as their energy source and upon activation switch to glycolysis (139–141). Glucose transporters GLUT1 and also GLUT3 and GLUT4 are rapidly induced and traffic to the cell membrane to promote glycolytic metabolism and cell growth (142). However, not all differentiating T cells have the same energetic requirements and in fact have quite distinct metabolic programs (143). Although T cells differentiating into Th1, Th2, and Th17 cells reprogram their metabolic pathways by turning on glycolysis, Michalek et al. first showed that differentiating Treg exhibit a unique metabolic profile relative to other CD4+ effector subsets. In this study, the Treg did not induce glucose transporters and upregulate glucose uptake and glycolysis; however, the Treg were not quiescent, and instead, their mitochondrial membrane potential increased, and lipid oxidation likewise increased. Insight into the Treg response to glycolysis, including transcriptional programming of splice variants of FOXP3 itself, has provided insight into loss of Treg function in autoimmunity and demonstrates that control of FOXP3 expression is important for stable suppressive function (144, 145). Recently, Weinberg et al. (146) have added to this, demonstrating that mitochondrial complex 3 is essential for Treg suppressive function. In mitochondrial complex 3-deficient mice, FOXP3 expression itself is not altered. However, immune regulatory gene expression and suppressive function were ablated. Fatty acid pathways have been linked to mitochondrial integrity, and this in turn impacts suppressive capability, and, for example, inhibition of the fatty acid binding protein 5 (FABP5) enhances suppressive function mediated by IFN1 signals and IL10 induction (147).

mTOR Signaling in CD4 Tconv and Treg

The kinase mTOR is activated upon CD4+ T-cell activation and has a pivotal role in the management of crucial cell functions, sensing a range of environmental cues such as cytokines, growth factors, and nutrients to regulate metabolism, protein synthesis, proliferation, and survival (148–150). mTOR signaling takes place via two complexes mTORC1 and mTORC2, whose regulation and activities are somewhat distinct from each other. An essential component of the mTORC1 complex is the scaffolding protein, regulatory associated protein of mTOR (RAPTOR). Activation of mTORC1 through PI3K-Akt signaling pathways has a central role in regulating T-cell growth and proliferation (148, 150) and has been shown to be required for correct differentiation of Th1 and Th17 cells, while mTORC2 and its essential subunit protein rapamycin-insensitive companion of mTOR (Rictor) are vital for Th2 differentiation (148–150). Activation of mTOR suppresses Treg development (151) while mTOR-deficient cells (148, 150) or cells with a blockade of glycolysis (152) differentiate into Treg. However, mTORC1 signaling is a pivotal positive determinant of Treg function under steady state and immune stimulation (153). mTORC1 activity coordinates the increase in CTLA4 and ICOS expression in Treg to upregulate their suppressive activity, orchestrating the lipogenic program. Raptor-deleted mice develop severe autoimmune disease, which demonstrates that this is absolutely required. There is hence a fundamental role for Raptor/mTORC1 in cholesterol and lipid biosynthesis, highlighting the mevalonate pathway as important for coordinating Treg proliferation and induction of effector molecules CTLA4 and ICOS. In addition, there is a role for liver kinase B1 (LKB1) in coordinating intracellular cholesterol biosynthesis via the mevalonate pathway in Treg cells, further demonstrating this pathway as crucial in the inhibition of inflammatory cytokine production and promotion of the suppressive activity of Treg (154). Thus, fine rheostat control of lipid metabolism is crucial for the optimal programming of suppressive activity, immune homeostasis, and immune tolerance in Treg cells (143). With the use of a knockout mouse model, the importance of these pathways in suppressor function was recently highlighted, demonstrating

that the absence of FOXP3 antagonism of mTOR promoted suppressor function (155).

Plasticity Driven by the Metabolic Program of Treg and CD4+ T Cells

Treg are clearly more pleiotropic than previously envisaged. Their metabolism may oscillate between mTOR-dependent and mTOR-independent pathways in response to environmental cues (144) depending on whether they are receiving signals to differentiate, proliferate, or carry out suppressive functions. The control of energy metabolism through the leptin–mTOR pathway in Treg sets their state of responsiveness, and this may be necessary for entry into the G1/S phase of cell cycle and proliferation. Recently, Pryadharshini et al. found that Treg metabolism is reprogrammed depending on whether the Treg are thymic derived (tTreg) or induced (iTreg). Inducible Treg are dependent on mitochondrial oxidative phosphorylation, whereby FOXP3 suppresses glycolysis. In contrast, tTreg engaged in glycolysis more comparable to that of effector T cells. Thus, the different Treg subsets utilized mTOR-dependent and mTOR-independent signaling pathways (156). Treg cells express several Toll-like receptors (TLRs), and these are critical for correct Treg homeostasis and function. Gerriets et al. (157) showed that as Treg proliferate, Glut1 levels increase, mTOR is activated, TLR1 and TLR2 are ligated, but at the same time FOXP3 is downregulated and suppressive activity reduced. Transgenic expression of Glut1 reduced Treg suppressive capacity and downregulated FOXP3. Conversely, FOXP3 diminished PI3K-Akt-mTOR signaling and Glut1 expression. Thus, TLR signals and FOXP3 counter-regulate Treg cell metabolism to balance proliferation and suppressive function. This is one mechanism by which a Treg homing to a site of inflammation is temporarily shut down if the local pathogen load (bacterial lipopolysaccharide level) is high, but once the TLRs are no longer engaged, the Treg population regains suppressor function. Shifting the balance of metabolic control can direct T-cell differentiation to specific lineages which can be part of normal immune control but in dysregulation can also lead to immune pathogenesis. FOXP3-deficient Treg acquire effector-like characteristics and lose suppressive function, dysregulating mTORC2 pathways and upregulating glycolysis. Deletion of an mTORC2-associated protein, Rictor, can re-establish partial Treg phenotype by restoring suppressive function to the impaired Treg (155). This restoration of Treg function opens up the possibility of reprogramming the metabolism of deficient Treg (such as in IPEX disease where mutations to FOXP3 are common) by targeting particular metabolic pathways (summarized in **Figure 4**).

Treg in the Peripheral Tissues

Tissue-resident Treg are found in almost all tissues including visceral adipose tissue (158) and skin and have unique transcriptional programs enabling them to home to and reside in these locations (159). Tissue Treg are frequently associated

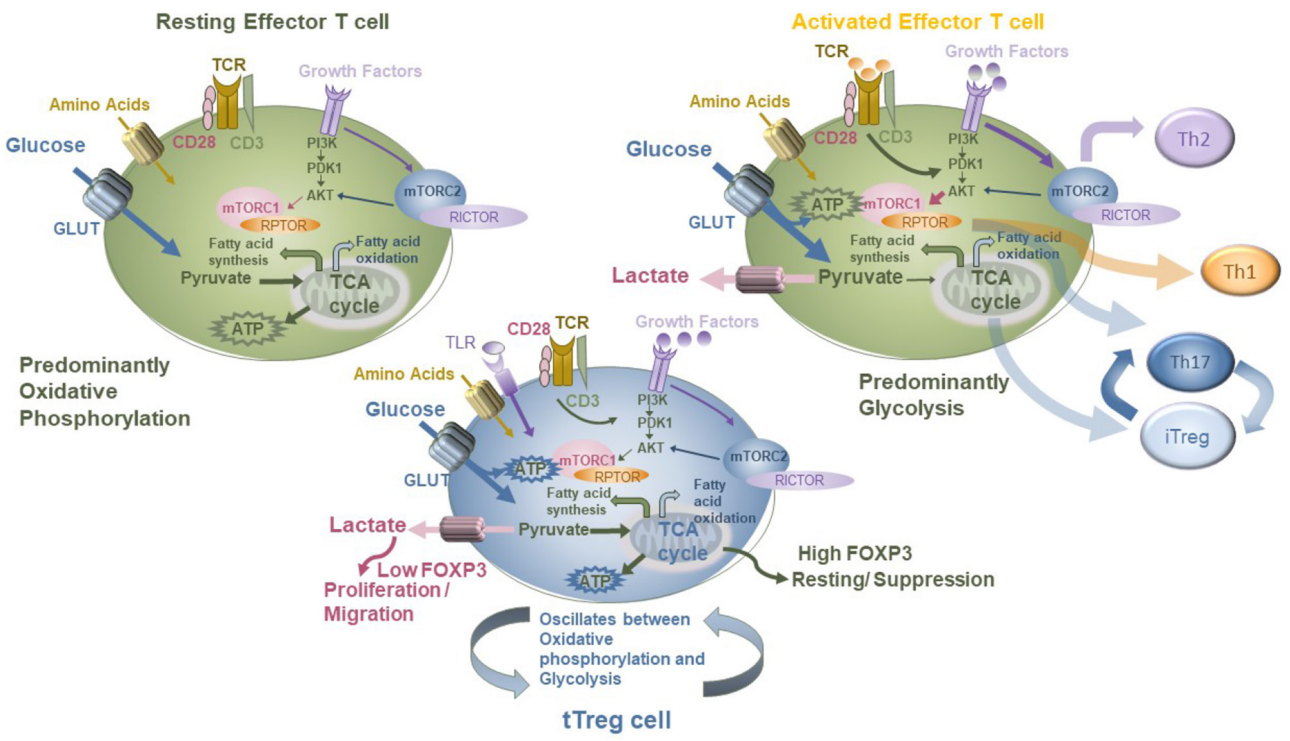


FIGURE 4 | Modeling the metabolic modifiers of T-cell function in Tconv and Treg, showing differential impacts of glycolysis and oxidative phosphorylation on each and the role of the mTOR pathway in mediating this.

with damage repair and are activated or expanded in response to damage. This is mediated by alarmin signaling and induction of tissue-specific tissue repair signals, e.g., amphiregulin. The IL33–ST2 signaling partnership is key to tissue Treg function, where local expression of IL33 or IL1 has opposing effects on Treg polarization and hence function (160–162), giving fine-tuning of Treg function in the tissue in response to injury. There is hence a growing role for tissue-resident Treg, such as VAT Treg (163); however, some of the tissue repair function is independent of suppressor function (7). This is worthy of fine analysis as localized inflammation is also suppressed by tissue Treg, but it is not yet clear if this is a heterogeneous mixture of subsets in the tissue or a dual function of a single subset (164). ST2+ Treg are implicated in tumor tolerance, and this may be an underappreciated consequence of their role in tissue repair (165). It is of some clinical relevance that tissue repair by Treg in zebrafish is able to reverse organ damage (166), but in the context of autoimmune damage in mice or humans, this capability is lost.

Treg and Immune Disease

It is possible that under specific circumstances, the balance of Treg to effector lineages may be altered or that the Treg reprogram and switch fates. Given the complexity and connectedness of the Treg GRN, there are many points that alter Treg or Tconv function, many of which could be affected by genetic or environmental risk factors. It is relatively rare to find mutations in FOXP3 itself (IPEX), suggesting that Treg-specific defects in autoimmune disease are likely to result from reduced FOXP3 function or alterations in expression of downstream targets, but not as a result of sequence changes in the FOXP3 gene body. This is further complicated by the observation that there are also genes involved in Treg function that are FOXP3 independent. It is interesting to note that when standard Treg flow cytometry data are analyzed using tSNE algorithms, three distinct FOXP3 populations can be resolved, and this is more complex than can be observed by 2D FACS analysis. Decreased Treg numbers or impaired Treg function in adult mice can cause autoimmune diseases (1), and the mechanisms and drivers of this have been revealed using numerous gene-targeting and fate-mapping models that also develop disease when the Treg compartment is perturbed. The therapeutic potential of inducing or restoring tolerance has also been demonstrated using adoptive transfer of Treg, which ameliorates many symptoms in non-obese diabetic (NOD) and IBD mouse models (1), as well as mouse models of pregnancy disorders, which mimic autoimmune disease in many regards (167). In humans, this is mirrored in IPEX patients who lack FOXP3 and Treg (28), and the early development of autoimmune disease in IPEX confirms that Treg are also essential in humans (168). Taken together, these data suggest that a threshold of Treg function is required throughout life to restrain autoreactive T cells and/or inflammatory responses, and loss control of this process licenses autoimmune disease onset. Nonetheless, there are conflicting reports in the literature about reduced Treg numbers in clinical autoimmune cohorts. This may be caused in part because of evolving biomarker combinations used and methods for enumerating Treg and impacted by the need to consider the amount of FOXP3 as well as the absolute presence or absence of FOXP3+ cells in flow cytometric data. This was also confounded by the observation that activation of T cells induces FOXP3 transiently in cells that are not Treg. We and others have demonstrated that loss of FOXP3 expression levels, rather than reduced absolute cell number, is observable in autoimmune cohort samples and may be a precipitating factor for reduced immune tolerance in these cohorts (24).

In Treg, a consequence of loss of FOXP3 expression as a result of transcriptional or translational defects could be reduced Treg function. This might be triggered by exhaustion or chronic overstimulation, such as could happen during a potent immune response, and this has led to the concept of ex-Treg. Fate-mapping studies elegantly demonstrate that, in mice at least, Treg can lose expression of FOXP3, but they are demonstrably of thymic Treg origin, based on genetic marking. These studies implicate ex-Treg in susceptibility to multiple sclerosis (169) and rheumatoid arthritis (170) and suggest that the high levels of IL6 at sites of tissue damage and inflammation can induce this loss of FOXP3 expression *in vivo*. As IL2 signaling is repressed by SOCS1 (171) and SOCS1 is induced by IL6 signals, this may be a contributing factor for reduced FOXP3 expression in pro-inflammatory scenarios. We and others have reported elevated IL6 and IL1, among other pro-inflammatory cytokines, in the local tissues in autoimmune disease samples including IBD (172, 173). Hence, a second axis driving plasticity may be the impact of local pro-inflammatory cytokines including IL6. Mechanistic insight into this has emerged recently with the identification of a huTreg subset that expresses gp130, the common gamma chain of the IL6 receptor, and the finding that these $gp130^{+}$ Treg are less suppressive and express lower levels of FOXP3 (174). The loss of tissue-resident Treg function is also associated with pathology and observed in almost any non-lymphoid tissue, and these include the lungs (175, 176), liver (177), and skin (178), and this is the result of either Treg intrinsic defects or altered IL33 signaling, such as that found in allergen-sensitive airways (176).

Environmental and Genetic Risks Combine to Alter Immune Function

The observation that many autoimmune diseases are the result of the intersection of genetic risk and external environmental triggers comes from the fact that there is incomplete penetrance in all autoimmune diseases, and this is in spite of the presence of genetic risk. Type 1 diabetes (T1D) is an example of a disease which arises as a result of complex interactions between genetic and environmental factors conspiring to drive the pathology, resulting in disease progression. In a meta-analysis of six independent genome-wide association studies (GWAS), each aiming to identify single-nucleotide polymorphisms (SNPs) that track with T1D, ∼45 loci were enriched (179, 180). We hypothesize that as the disease linked to a failure of self-tolerance, Treg cells would in some way be impacted by this genetic risk, so we intersected the genetic risk loci with our FOXP3 ChIP data (57). This revealed that 34 (>70%) contain

a FOXP3-binding site, which is significantly above the genome-wide distribution of FOXP3-binding sites (∼15%). Hence, the enrichment of T1D genetic risk in regions that are potentially directly controlled by FOXP3, and therefore actively regulated in Treg, suggests potential for a Treg-specific defect directly as a result of polymorphism in regulatory elements controlling these genes.

As described above, genome-wide mapping of epigenetic variation in Treg and Tconv suggests the potential for cell-type-specific transcriptional activity, but this is not restricted to T1D (181, 182). From numerous GWAS datasets, there is very strong probability data linking genetic variation (SNP) to a wide variety of immunological disease cohorts. It is clear that the majority of this variation is not in the coding regions of the genome but in specific non-coding regions enriched for regulatory elements such as promoters and enhancers (101). When this is nuanced with datasets that have functional annotation, the majority of this non-coding genetic variation overlaps and thus likely influences transcription factor binding sequences and lncRNAs (183–185). Therefore, the functional impact of genetic risk has to be studied in the cell type driving diseases. It is not possible to accurately define this in cell lines from other tissues. With regard to GWAS datasets derived from autoimmune cohorts, the significant enrichment in T-cell-specific promoters and enhancers (100, 185) suggests that perturbation of gene regulation in multiple pathways in the immune system can result in the same phenotype from unrelated genotypes.

As this is a bioinformatics-based intersection, the functional link between them is currently unknown. The same genetic risk is carried in all CD4 effector T-cell populations, and although many cell types contribute to immune homeostasis, Treg/Tconv defects play a major role in the pathology of human autoimmune disease. Thus, it is plausible that both effector and Treg are impacted by genetic variation. Susceptibility to disease is therefore linked to Treg plasticity, altered Treg development, or altered Treg function, and this can be therapeutically targeted once the pathways are identified. For example, TNF antagonism has been demonstrated to be effective on Treg in rheumatoid arthritis (186).

CONCLUSION AND FUTURE DIRECTIONS

There are complex dynamic regulatory processes controlling Treg generation and stability that involve the interplay between transcription factors, miRNAs, and lncRNAs to shape Treg-specific regulation of gene expression. These occur between genes and enhancers over long and short distances and are only active in regions of open chromatin. Given that master transcription factors shape almost every lineage in the lymphoid compartment, it is plausible that these interact to set the expression levels of the transcription factors which themselves define function. Importantly, as T-cell function is dynamic and responsive to external cues, the enhancers and super-enhancers establish the level and kinetics of gene expression both in the steady state and in response to these cues. This gives three layers of reinforcement of Treg phenotype in the normal context. Each may be a point of disruption, either in disease, which alters the numbers or function of the Treg pool, or in response to appropriate tissue and inflammatory cues, which alters cell fate transiently. If this is a programmed change rather than induced by disease risk, it may be described as plasticity. Plasticity is hard to demonstrate in humans, as it is difficult to accurately model human T-cell fate in real time (**Figure 5A**).

Given that FOXP3 establishes and maintains a strong regulatory phenotype in healthy individuals that is resistant to reprogramming, there is potential for this to be disrupted under specific circumstances, e.g., in carriers of disease-associated genetic risk. The result could be the formation of a cell with an effector-like function that expresses less or no FOXP3. It is also plausible that the organ-specific damage in autoimmune diseases such as T1D may be a result of the dual impact of the loss of suppressor function resulting in an inappropriate anti-self-immune response, as well as a failure of the tissue repair capacity of the Treg, resulting in loss of beta cells (**Figure 5B**). However, using the newest genomics and high-resolution cell phenotyping, the question of identifying mechanisms underpinning loss of function in human Treg will likely soon be answered. This will necessarily require functional validation in human T-cell subsets. Methods including gene editing are a powerful tool for

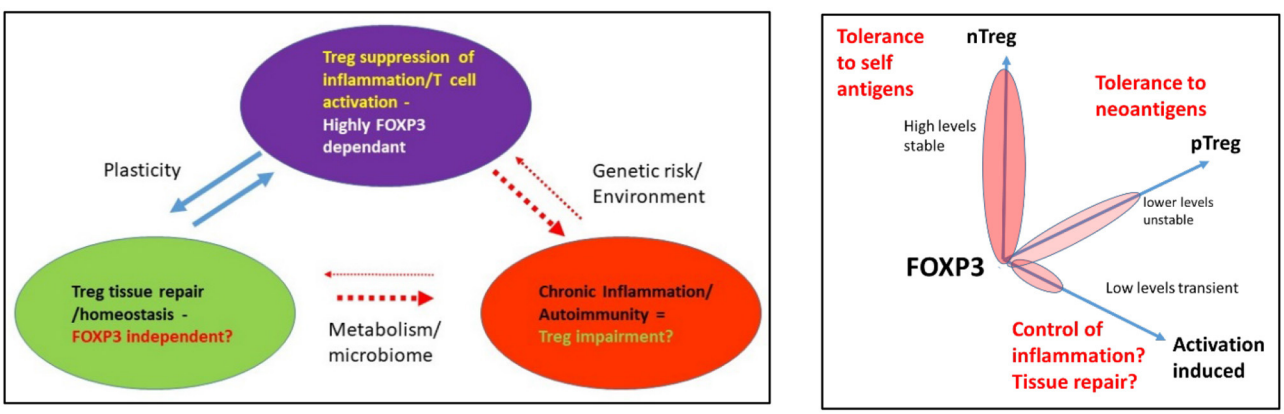


FIGURE 5 | (A) Functional roles of Treg and the impact of environment and genetic risk. **(B)** Modeling a functional link between FOXP3 levels and biological process.

pathway functional validation, including engineering of genetic risk into healthy T cells to assess its impact. It can also be used to target miRNAs and lncRNAs to assess their role in fine-tuning alterations in genotypes required to alter phenotypes and to perhaps confirm these are the rheostat of fate. This will also reveal if dysregulation of miRNAs is a tipping point for altered phenotypes. In time, these approaches will provide diagnostic information and new points for therapeutic intervention to reverse the impact of genetic risk on gene expression in Treg and Tconv in many diseases, including autoimmunity.

AUTHOR CONTRIBUTIONS

CB and TS contributed to writing, editing, and performed some of the work referred to in this review. YW, SW, NL, HW, KB, VB, and CH contributed to editing and figures generated for this review. SP and JB contributed bioinformatics analysis, editing, and experimental design input to this review. SB, MB, AF, and SR conceived, contributed to writing, and edited the work. All authors contributed to the article and approved the submitted version.

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